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14. ABSTRACT
Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, with many traditional antimicrobial agents becoming ineffective. Our main hypothesis is that predatory prokaryotes could serve as a novel therapeutic agent to control wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to prey on a wide range of pathogens including bacteria isolated from Wounded Warriors. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria and investigating predator prey interactions and resistance. Using enrichment culturing techniques we have verified that no genetically stable predation resistant phenotype developed in host cells following sequential predation. Our data also confirmed that the predators do not breach their host specificity and attack previously resistant bacteria. Cell toxicity assays, using human cell lines, demonstrated that predatory bacteria are significantly less toxic than the control. Finally, using a mouse wound model we determined that administering live predatory bacteria into exposed wounds did not cause any significant adverse effect to the animal or alter wound clinical score compared to a non-infected wound.

15. SUBJECT TERMS
Wounded soldiers, Predatory bacteria, Bio-control, Wound infections, Multi-drug-resistant, Bdellovibrio bacteriovorus, Micavibrio aeruginosavorus, Biofilms.

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Introduction

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, while many traditional antimicrobial agents are becoming ineffective. Resistance can be considered as a natural response to the selective pressure of a drug and can develop in both free-floating bacteria, as well as, in surface-attached bacteria or biofilms. One of the major difficulties in controlling surface-attached bacteria is their enhanced resistance to antimicrobial agents i.e. biofilm bacteria can be up to 1000 times more resistant to antimicrobial agents than their planktonic counterparts. Thus, the high doses of antimicrobials required to rid wounds and medical devices of biofilms are impractical. The problem of multidrug-resistant (MDR) bacterial infections in the Wounded Warrior drove researchers to examine other potential anti-bacterial strategies. Among these alternative therapies is the use of biological control agents such as medical maggots, phage, biodebridement, and predatory bacteria.

Our main hypothesis is that predatory prokaryotes are able to serve as a novel topical therapeutic agent in controlling non-treatable, wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria are able to prey on a wide range of pathogens including multidrug resistant bacteria isolated from Wounded Warrior. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria in *ex vivo* and *in vivo* systems. The proposal is divided into three aims: (I) Investigating predator-prey/host bacteria interactions and resistance, (II) Determining the effect of predatory bacteria on mammalian cells, and (III) Measuring the efficacy of predation and toxicity in animal models.

Body

Aim-I of the proposed study was to investigate predator-prey/host bacteria interactions and resistance. The goal of this task was to examine key questions regarding adaptation of the host to the predator. The aim is divided into three subtasks:

Subtask 1.1. Development of genetically stable resistance to predation.
Subtask 1.2. Examine the ability of the predator to breach its host specificity and attack previously resistant bacteria.
Subtask 1.3. Enrich for hyper predatory variants.

Aim I. Investigating predator prey interactions and resistance.
The goal of this task was to investigate key questions regarding adaptation of the host to predation.

Subtask 1.1. Development of genetically stable resistance to predation.
Rationale. It is believed that, unlike antibiotics or phage therapy, the selective pressure of predation does not generate genetically stable resistant variants in the host. Since the appearance of host resistance might reduce the efficacy of predation, we conducted experiments aimed at increasing the selective pressure on the host and assessing if any genetically stable predation resistant phenotypes emerge.

Aim-1, Task-1, Subtask 1.1, Experiment 1. Enriching for host resistant phenotypes by culturing.

Host bacteria were cultured with the predator for 24 hrs (predation cycle), thereafter, the remaining host cells were collected by centrifugation, suspended in predator-free media and allowed to grow for an additional 24 hrs (growth cycle). The host cells were collected once more and fresh predators were added (predation cycle). The predation and growth cycle were repeated 20 times. Finally, the reduction in total host was evaluated by CFU enumeration and compared to the initial host reduction measured during the first predation cycle.

Experiment 1.1.1
In these experiments, we have sequentially cultured host bacteria with two predators, *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. The host bacteria used in the experiment was *A. baumannii* NCIMB 12457. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction ($\log_{10}$) of *A. baumannii* after co-culturing with *B. bacteriovorus* HD100, *B. bacteriovorus* 109J or predator free control.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Initial average log change	+0.2	-2.3	-4.3

Final predation

Final average population reduction ($\log_{10}$) of *A. baumannii* that was sequentially cultured 20 times on **B. bacteriovorus HD100**.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.2	-3.6	-3.7

Final average population reduction ($\log_{10}$) of *A. baumannii* that was sequentially cultured 20 times on **B. bacteriovorus 109J.**

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.2	-3.8	-3.6

Results. From the data above, it seems that a stable resistant phenotype didn't develop on *A. baumannii* cells which were sequentially cultured on *B. bacteriovorus* 109J or HD100.

Experiment 1.1.2
In these experiments, we have sequentially cultured host bacteria with two predators, *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. The host bacteria used in this experiment was *K. pneumoniae* ATCC 33495. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction of *K. pneumoniae* ($\log_{10}$) after co-culturing with *B. bacteriovorus* HD100, *B. bacteriovorus* 109J or predator free control.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Initial average log change	+0.1	-5	-2.7

Final predation

Final average population reduction ($\log_{10}$) of *K. pneumoniae* that was sequentially cultured 20 times on **B. bacteriovorus HD100**.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	-0.15	-4.4	-3.5

Final average population reduction ($\log_{10}$) of *K. pneumoniae* that was sequentially cultured 20 times on **B. bacteriovorus 109J.**

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.1	-5	-2

In order to confirm that genetically stable predation resistant phenotype did not develop, cultures from the final *B. bacteriovorus* 109J enrichment experiment were stored and re-examined in predation experiments.

Reduction of *K. pneumoniae,* that was sequentially cultured 20 times on *B. bacteriovorus* HD100, and stored.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	0	-4.4	-2.7

Reduction of *K. pneumoniae,* that was sequentially cultured 20 times on *B. bacteriovorus* 109J, and stored.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	0	-5.0	-3.9

The data confirms that no stable resistance developed in *K. pneumoniae* following predation.

Results. The data obtained suggests that no genetically stable predation resistant phenotype developed in *K. pneumoniae* following sequential predation by *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. Furthermore, no genetically stable predation resistant phenotypes developed in *A. baumannii* following sequential predation by *B. bacteriovorus* HD100 and *B. bacteriovorus* 109J.

Aim-1, Task-1, Subtask 1.1, Experiment 1.1.3
In this experiment, we have sequentially cultured host bacteria with *M. aeruginosavorus*. The host bacteria used in this experiment was *P. aeruginosa* Pa14. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction of *P. aeruginosa* Pa14 after co-culture with *M. aeruginosavorus* or predator free control.

	Control	*M. aeruginosavorus*
Initial average log change	+0.1	-2

Reduction of *P. aeruginosa* Pa14 after 15 cycle of passage with *M. aeruginosavorus* then co-cultured with *M. aeruginosavorus* or predator free control:

	Control	*M. aeruginosavorus*
Average log change	-0.3	-2.3

Results: From the above data it seems *P. aeruginosa* Pa14 did not develop resistance to predation by *M. aeruginosavorus*.

Aim-1, Task-1, Subtask 1.1, Experiment 2. *Enriching for biofilm predation resistant phenotypes.*

Objective. The aim of this task was to see if biofilm resistant phenotypes develop in biofilm that were cultured multiple times in the presence of the predator.

Bacteria, grown as biofilms, were sequentially grown in the presence of the two predators, *B. bacteriovorus* 109J and predator *B. bacteriovorus* HD100. The host bacteria used in this experiment was *A. baumannii* NCIMB 12457. The experiment was conducted in 8 wells for each treatment and examined for the emergence of a predation resistant biofilm. CV staining was used to quantify biofilm reduction due to predation.

The data below represents the biofilm reduction in host cells in the initial co-culture.
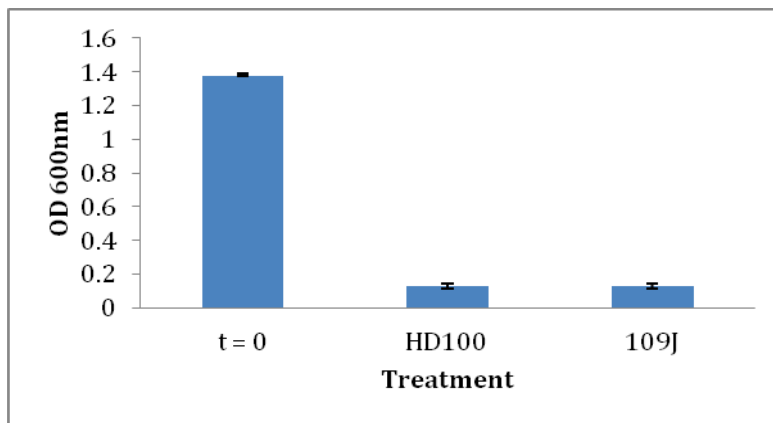
The data below represents the biofilm reduction of host cells that were sequentially cultured 7 times on *B. bacteriovorus* HD100 and then exposed to the predators.
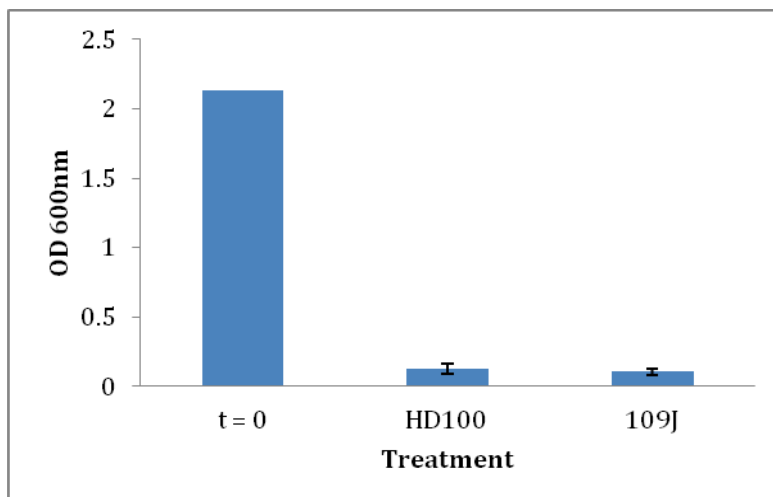
The data below represents the biofilm reduction of host cells that were sequentially cultured 7 times on *B. bacteriovorus* 109J and then exposed to the predators.
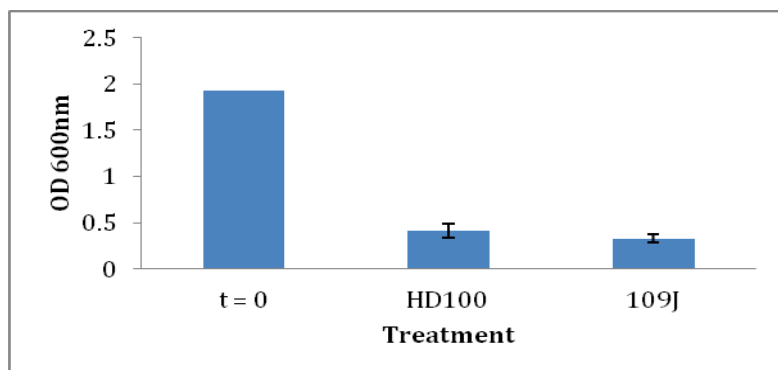

Results. The data obtained suggests that host cells grown as a biofilm do not adapt to form predation resistant biofilms.

Aim-1, Task-1, Subtask 1.1, Experiment 3. *Isolating host resistant mutants by induced mutagenesis.*

As culture selection alone did not impose sufficient selective pressure for inducing host resistant phenotypes, we attempted to produce predation resistant mutants by random mutagenesis. UV radiation and transposon mutagenesis were used to introduce mutations. Thereafter, the mutants were evaluated for the emergence of predation resistance. In this set of experiments *K. pneumoniae* ATCC 33495 was mutagenized and used as host for both *B. bacteriovorus* 109J and *M. aeruginosavorus*.

<u>Outcome</u>. At the completion of growth cycle 7 (out of 10) we could conclude that no predation resistant phenotypes were detected in *K. pneumoniae* following sequential predation with *B. bacteriovorus* 109J and *M. aeruginosavorus*.

Subtask 1.2. Investigating the ability of the predator to breach its host specificity and attack previously resistant bacteria.

<u>Rationale.</u> We have previously conducted experiments aimed at investigating the host range of each predator. However, it could be speculated that during the predation process alterations might cause a change in host specificity. Although a breach in host specificity could be less desirable, as it might allow the predators to attack communal non-pathogenic Gram-negative bacteria, it could be favorably used in order to increase the predatory portfolio of the predator

allowing it to attack new desired pathogens. In the following sets of experiments we investigate if a breach or alteration in predator host specificity could arise.

Subtask 1.2. Experiment 1. *Enriching for B. bacteriovorus 109J with altered host specificity.*

The aim of this experiment was to investigate if *B. bacteriovorus* will breach its host specificity and attack a host that initially was resistant to predation. To this end, *B. bacteriovorus* was co-cultured with two hosts *Stenotrophomonas maltophilia* ATCC 13636 and *Streptococcus epidermidis* ATCC 12228. The inability of the predators to prey on the host was confirmed (Table-1 and 3). Thereafter, we have attempted to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria (Table-2 and 4).

B. bacteriovorus 109J was suspended in a culture containing a 1:1 ratio of the non-host bacteria (*S. maltophilia* or *S. epidermidis*) and the host bacteria *E. coli* strain WM3064, a diaminopimelic acid (DAP) auxotroph. The specific *E. coli* was used in order to allow initial *Bdellovibrio* cell growth. After 48 hrs of predation, the *Bdellovibrio* was isolated by filtration (0.45 um filter) and re-cultured in a new co-culture containing a 2:1 ratio of non-host and host (predation resistant bacteria). The predation cycles were repeated 11 times, in each cycle the fraction of the host *E. coli* was reduced. Finally, *Bdellovibrio* cells were isolated and their ability to prey on *S. maltophilia* (Table-1 and 2) or *S. epidermidis* (Table-3 and 4) was examined. All experiments were conducted in triplicates. Data represent the average log change.

Table-1. Initial reduction of *S. maltophilia* after co-culturing with *B. bacteriovorus* 109J.

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.2	-0.2

Table-2. Final population reduction of *S. maltophilia* exposed to predator-free control and the predator *B. bacteriovorus* 109J that was sequentially cultured with *S. maltophilia*.

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.11	+0.11

Table-3. Initial reduction of *S. epidermidis* after co-culturing with *B. bacteriovorus* 109J.

	Control	*B. bacteriovorus* 109J
Final average log change	-0.41	-0.3

Table-4. Final population reduction of *S. epidermidis* exposed to predator-free control and the predator *B. bacteriovorus* 109J that was sequentially cultured with *S. epidermidis*.

	Control	*B. bacteriovorus* 109J
Final average log change	-0.55	-0.55

Result: The data obtained suggests that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria.

Subtask 1.2. Experiment 2. *Enriching for M. aeruginosavorus with altered host specificity.*

The aim of this experiment was to investigate if sequential re-culturing of *M. aeruginosavorus* ARL-13 on a non-host bacteria will result in predation. To this end, *M. aeruginosavorus* was co-cultured with two hosts *Stenotrophomonas maltophilia* ATCC 13636 and *Streptococcus epidermidis* ATCC 12228. The inability of the predators to prey on the host was confirmed (Table-1A and 2A). Thereafter, we have attempted to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria (Table-3A and 4A).

M. aeruginosavorus was suspended in a culture containing a 1:1 ratio of the non-host bacteria (*S. maltophilia* or *S. epidermidis*) and the host bacteria *E. coli* strain WM3064, a diaminopimelic acid (DAP) auxotroph. The specific *E. coli* was used in order to allow initial *Micavibrio* cell growth. After 48 hrs of predation the *Micavibrio* lysate was re-cultured in a new co-culture containing a 2:1 ratio of non-host and host (predation resistant bacteria). The predation cycles were repeated 11 times, in each cycle the fraction of the host *E. coli* was reduced. Finally, *Micavibrio* cells were isolated and their ability to prey on *S. maltophilia* (Table-1 and 2) or *S. epidermidis* (Table-3 and 4) was examined. All experiments were conducted in triplicates. Data represent the average log change.

Table-1A. Initial reduction of *S. maltophilia* after co-culturing with *M. aeruginosavorus*.

	Control	*M. aeruginosavorus*
Initial average log change	+0.1	+0.1

Table-2A. Final population reduction of *S. maltophilia* exposed to predator-free control and the predator *M. aeruginosavorus* that was sequentially cultured with *S. maltophilia*.

	Control	*M. aeruginosavorus*
Initial average log change	-0.4	-0.4

Table-3A. Initial reduction of *S. epidermidis* after co-culturing with *M. aeruginosavorus*.

	Control	*M. aeruginosavorus*
Final average log change	-0.3	-0.3

Table-4A. Final population reduction of *S. epidermidis* exposed to predator-free control and the predator *M. aeruginosavorus* that was sequentially cultured with *S. epidermidis*.

	Control	*M. aeruginosavorus*
Final average log change	-0.33	-0.2

Result: The data obtained suggests that *M. aeruginosavorus* does not have an ability to breach its host specificity and attack previously resistant bacteria.

Subtask 1.3. Enriching for hyper predatory variants.
Rationale. As the long-term goal of our study is to develop predatory bacteria as a topical bio-control agent, isolating superior predatory isolates is desired. The aim of this task is to enrich for predator variants that exhibit an elevated predation phenotype.

Experiment 1. *Enriching for hyper virulent B. bacteriovorus.* Previous experiment showed that *B.bacteriovorus* 109J was able to reduce *A. baumannii* strain AB3917 and AB5256 by a **single log,** as opposed to a 5-log reduction seen on most isolates. The aim of this task was to sequentially culture the predator on each of the host in order to enrich for hyper virulent predators.

B. bacteriovorus 109J was co-cultured with *A. baumannii* AB3917 and *A. baumannii* AB5256 for 48 hrs and the reduction of host bacteria was measured (Tables 5 and 5A). After 48 hrs the *Bdellovibrio* was isolated by filtration (0.45um Milex) and re-cultured with fresh *A. baumannii*. As a control, *Bdellovibrio* was cultured with *E. coli*. The predation cycles were repeated 10 times. The predation ability of the culture-enriched *Bdellovibrio* was compared to a control *Bdellovibrio*, which was not co-cultured with *A. baumannii*. All experiments were conducted in triplicates. Data represent the average log change.

Table 5. Initial reduction of *A. baumannii* AB3917 after co-culture with *B. bacteriovorus* 109J or predator free control:

	Control	*B. bacteriovorus* 109J
Initial average log change	-0.3	-5.0

** The outcome of this experiment is somewhat unexpected, as we initially have seen only a one log reduction in the ability of *B. bacteriovorus* 109J to reduce *A. baumannii* AB 3917. We could only speculate that the ability of *B. bacteriovorus* 109J to reduce *A. baumannii* AB 3917 altered during storage of the predator in the lab and continuous passing of the predator.

To measure if we can enrich for hyper virulent *B. bacteriovorus*, the 109J strain was cultured on *A. baumannii* AB3917. As seen in Table 5 a slight increase in the ability of *B. bacteriovorus* to reduce AB3917 was seen.

Table 5a. Final reduction of *A. baumannii* AB3917 after co-culturing with predator free control and *B. bacteriovorus* 109J that was sequentially cultured on AB3917.

	Control	*B. bacteriovorus* 109J
Final average log change	+0.1	-5.5

Enriching for hyper virulent *B. bacteriovorus* on *A. baumannii* AB5256

To measure if we can enrich for hyper virulent *B. bacteriovorus*, the 109J strain was cultured on *A. baumannii* AB5256.

Table 6. Initial reduction of *A. baumannii* AB5256 after co-culturing with *B. bacteriovorus* 109J or predator free control:

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.2	-0.9

Table 6a. Final reduction of *A. baumannii* AB5256 after co-culturing with predator free control and *B. bacteriovorus* 109J that was sequentially cultured with AB5256.

Experiment	Control	*B. bacteriovorus* 109J
Final average log change	-0.1	-0.6

Result: The data above shows that after continuous predation cycles there were no increase in the ability *B. bacteriovorus* 109J to reduce this specific strain.

Additional experiment.
Since *B. bacteriovorus* 109J that was cultured on *A. baumannii* AB5256 did not become more aggressive, we were interested in measuring if *B. bacteriovorus* 109J that was passed on AB3917 or *E. coli* will be more aggressive against this strain. Predation experiments were performed using *A. baumannii* AB5256 as host and *B. bacteriovorus* 109J that was passed on AB5256, AB3917 and *E. coli* (Table 7, 7a and 7c respectively) as predator.

Table 7. Reduction of *A. baumannii* AB5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with AB5256:

Experiment	Control	*B. bacteriovorus* 109J
Average reduction log change	-0.1	-0.9

Table 7a. Reduction of *A. baumannii* AB5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with AB3917.

	Control	*B. bacteriovorus* 109J
Average reduction log change	+0.5	-1.2

Table 7c. Reduction of *A. baumannii* 5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with *E. coli*.

	Control	*B. bacteriovorus* 109J
Average reduction log change	+0.09	-1.2

Result: The data obtained suggests that *B. bacteriovorus* 109J could become more virulent on a particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria and not others.

Subtask 1.3. Experiment 2. *Enriching for hyper virulent M. aeruginosavorus.* Previous experiments showed that *M. aeruginosavorus* ARL-13 was able to reduce *A. lwoffii* strain ATCC15309 by less than a log. The aim of this task was to sequentially culture the predator on each of the hosts in order to enrich for hyper virulent predators.

M. aeruginosavorus ARL-13 was co-cultured with *A. lwoffii* strain ATCC15309 for 48 hrs and the reduction of host bacteria was measured (Tables 8 and 8a). After 48 hrs the *M. aeruginosavorus* was isolated by filtration (0.45um Milex) and re-cultured with fresh *A. lwoffii*. The predation cycles were repeated 12 times. The predation ability of the culture-enriched *M. aeruginosavorus* was compared to a control *M. aeruginosavorus*.

Table 8. Initial reduction of *A. lwoffii* after co-culturing with *M. aeruginosavorus ARL-13* or predator free control:

	Control	*M. aeruginosavorus*
Initial average reduction log change	+0.2	-0.3

Table 8a. Final reduction of *A. lwoffii* after co-culturing with *M. aeruginosavorus* ARL-13 or predator free control:

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	Control	*M. aeruginosavorus*
Final average reduction log change	0	-0.24

Result: The data above shows that after continuous predation cycles there were no increase in the ability *M. aeruginosavorus* to reduce *A. lwoffii*.

Experiment 3. *Enriching for predatory bacteria variant more efficient at predation at elevated temperatures.*

The aim of this experiment was to enrich for high temperature predation variants more suitable for medical application. To this end, predation of *Bdellovibrio* and *Micavibrio* at three different temperatures (30°C, 37°C and 39°C) was evaluated.

We observed that predators showed significant host reduction at temperatures 30°C and 37°C but not at 39°C. Hence, enrichment experiment was done using *B. bacteriovorus* 109J lysate and *Micavibrio* lysate from 37°C and then repeated several predation cycles after every 48 hrs until the temperature reached 39°C. In each cycle the incubation temperature was increased by 0.5°C until the temperature reaches 39°C. The predators from the last cycle were isolated and their ability to prey at 39°C was measured.

In this experiment predation was measured by the reduction in culture turbidity using Synergy H1 Hybrid Reader.

The following Table-9 shows the average reduction in culture turbidity. Co-cultures were placed at 39°C, the highest temperature reached during the experiment. The following predators were used:
Sample A: Non temperature acclimated *Bdellovibrio*.
Sample B: Acclimated *Bdellovibrio*.
Sample C: Non temperature acclimated *Micavibrio*.
Sample D: Acclimated *Micavibrio*.

Table 9: Culture turbidity change. Data represent the average of three experiments.

Predator used	Non temperature acclimated *Bdellovibrio*	Acclimated *Bdellovibrio*	Non temperature acclimated *Micavibrio*	Acclimated *Micavibrio*
Average change in culture turbidity	3.6± 3%	43±9%	14±9%	37±5%

Summary: The data obtained suggests that *Bdellovibrio* 109J and *Micavibrio* could be acclimated to prey at an elevated temperature of 39°C.

Additionally we ran the temperature predation experiment with both *Bdellovibrio* 109J and *Micavibrio* using the Synergy H1 Hybrid Reader. *Bdellovibrio* 109J and *Micavibrio lysates were* prepared and incubated in the spectrophotometer at 39°C for 80 hrs. The change in culture turbidity was measured.
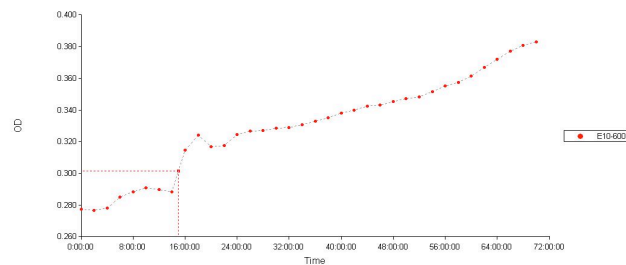
The predators used were:
Sample A: Non temperature acclimated *Bdellovibrio*.
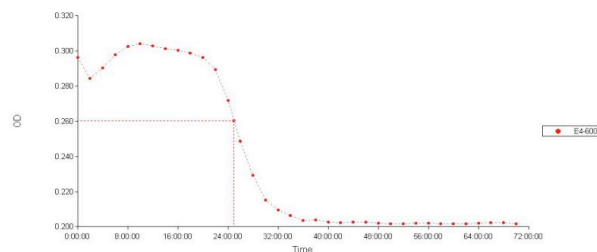Sample B: Acclimated *Bdellovibrio*.

Sample C: Non temperature acclimated *Micavibrio.*
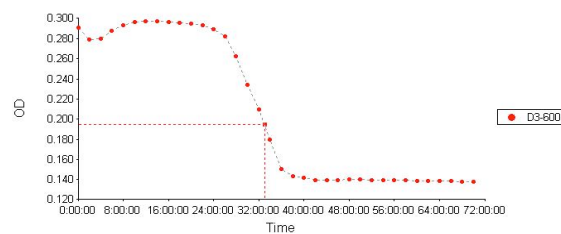Sample D: Acclimated *Micavibrio*.

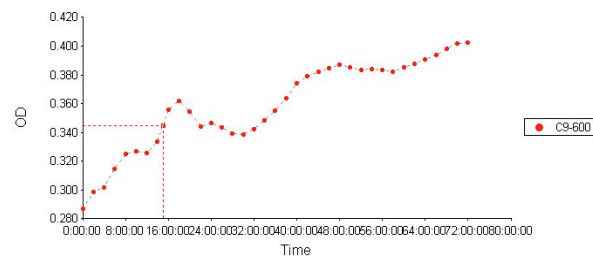**Fig 1: *K. pneumoniae* predator-free control incubated at 39°C.**

Fig 2: *K. pneumoniae* incubated with a non-temperature acclimated *B. bacteriovorus 109J* set at 39°C.
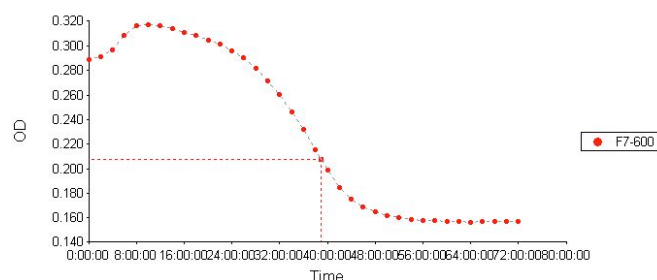

Fig 3: *K. pneumoniae* incubated with a temperature acclimated *B. bacteriovorus 109J* set at 39°C.

Fig 4: *K. pneumoniae* incubated with a non-temperature acclimated *M. aeruginosavorus* set at 39°C.

Fig 5: *K. pneumoniae* incubated with a temperature acclimated *M. aeruginosavorus* set at 39°C.

Result: The data suggests that *B. bacteriovorus* 109J as well as *M. aeruginosavorus* could be acclimated to prey at elevated temperature of 39°C.

Aim II. Determining the effect of predatory bacteria on eukaryotic cells. Although the effect of predation on prokaryotic Gram-negative cells is documented, limited data is available regarding predation on eukaryotic cells. As the goal of our research is to utilize predatory bacteria as a live antibiotic, examining the potential risk of predatory bacteria on non-microbial host is essential for the development of a safe bio-control agent.

The goal of this aim is to determine if predatory bacteria have an adverse affect on eukaryotic cells. The aim is divided into three subtasks.

Subtask 2.1. *Determining the toxicity of predatory bacteria on mammalian cells.*
Subtask 2.2. *Determine the influence of predator exposure on cell cytokine profile.*
**Subtask 2.3.** *Cell attachment and invasion assay.*

Subtask 2.1. *Determining the toxicity of predatory bacteria on mammalian cells.*
Rationale. It was previously demonstrated that, when added to human cell lines, some Gram-negative bacteria could induce cell death. In order to investigate whether predatory bacteria have an adverse toxic affect on eukaryotic cells we will introduce predatory bacteria to mammalian cell cultures and examine the change in cell viability.

The effect of *B. bacteriovorus* 109J and HD100 on mammalian cell viability was investigated. Initial experiments were conducted with 3 different concentrations of predatory bacteria following 4 and 24 hrs of exposure. Each experiment was conducted on 2 selected cell lines; HaCAT human epidermal keratinocytes, and NCTC human fibroblasts. Three experiments were conducted. The effect of predatory bacteria on mammalian cell viability will be examined using PrestoBlue™ cell viability reagent.

Outcome. The data below represent the result of the cell toxicity assays. The data represents the average and SD of 9 replicates from 3 separate experiments. The data obtained suggests that *B. bacteriovorus* 109J and HD100 are significantly less toxic than the *Pseudomonas aeruginosa* used as control (10^9 CFU).
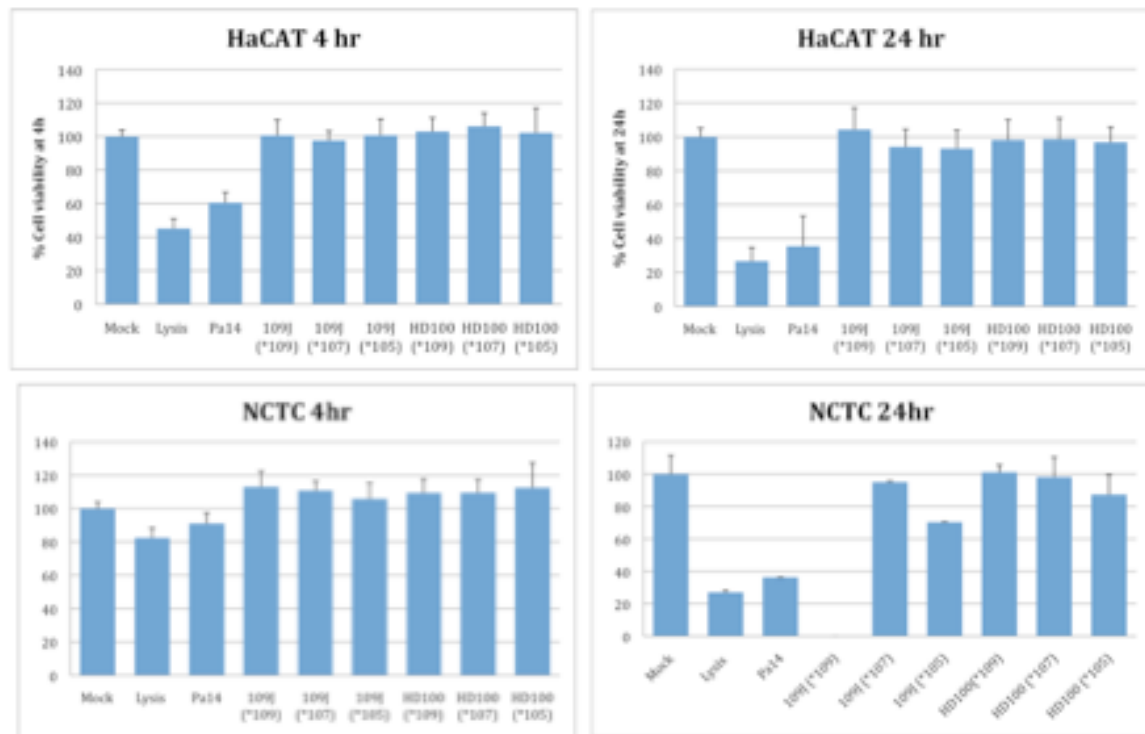
Since we did not see any significant killing by the predators, we continued the experiment using only the high cell density of the predatory bacteria.

In the following experiment 3 predatory bacteria (1×10^9) were co-cultured with the three selected cell lines. HaCAT human epidermal keratinocytes, Hs27 human skin fibroblasts and L929 murine fibroblasts.

Outcome. No statistical significant reduction in cell viability was observed when predatory bacteria (1×10^9) were co-cultured with any of the examined cell line. The data below represent the result of the cell toxicity assay. The data represents the average and SD of 12 replicates from 4 separate experiments. The data obtained suggests that *B. bacteriovorus* 109J and HD100 and *Micavibrio* are significantly less toxic than the *P. aeruginosa* (10^9 CFU) used as control.

HaCAT 4 hrs

HaCAT 24 hrs

Hs27 4 hrs

Hs27 24 hrs
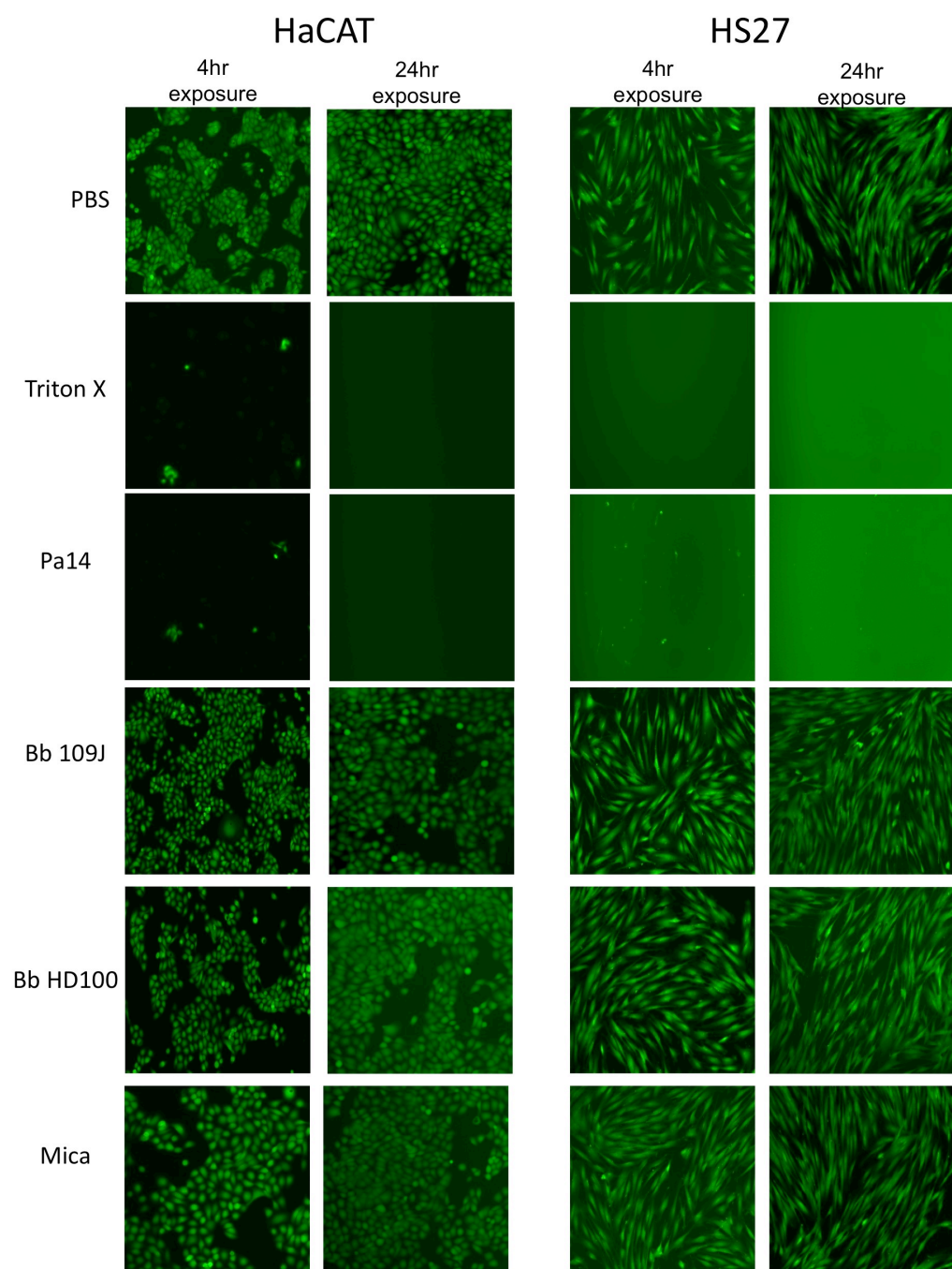

L929 4 hrs

L929 24 hrs
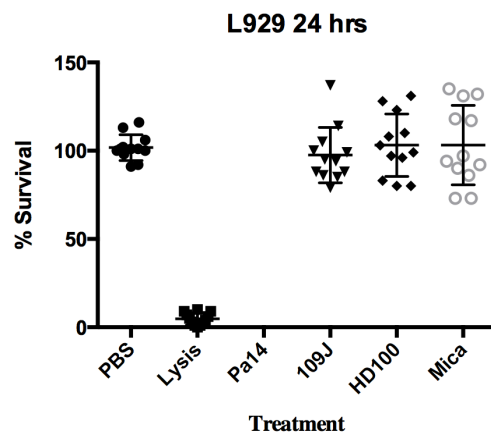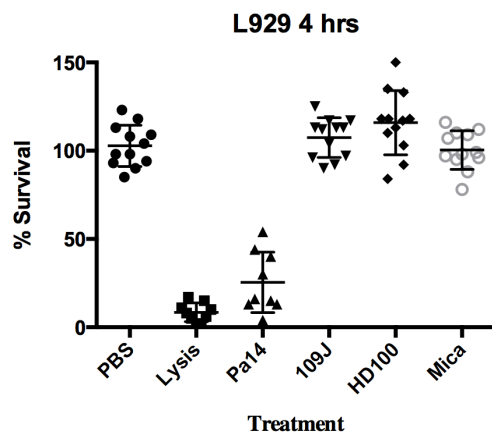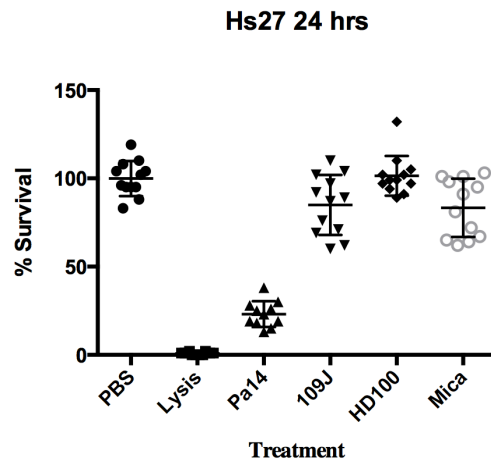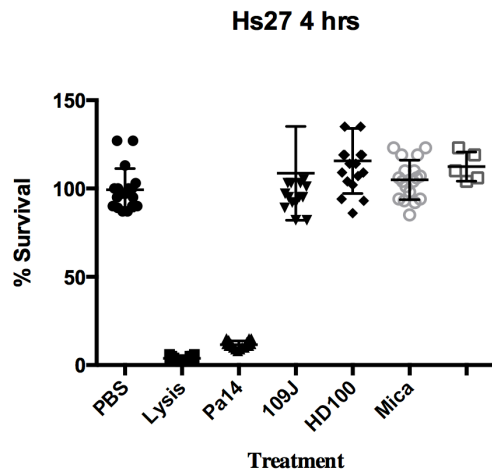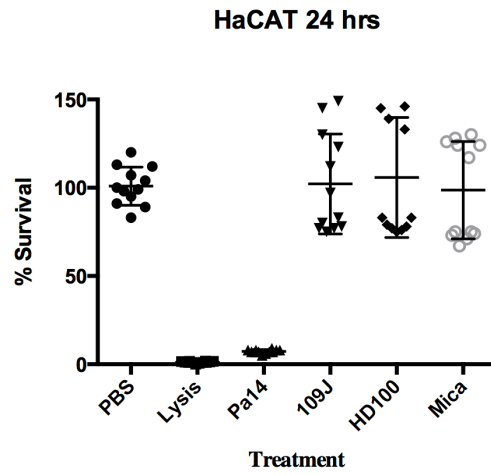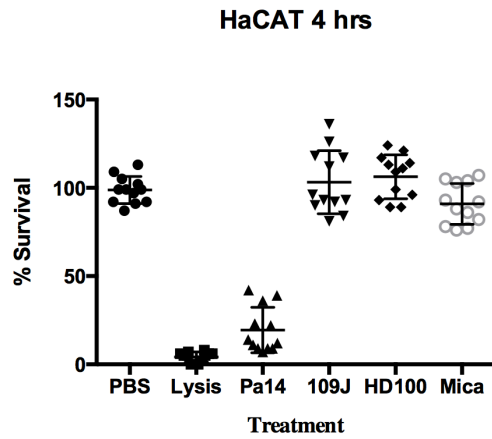
Conclusion. Predatory bacteria do not have a negative effect on eukaryotic cells viability when examined ex-vivo.

Imaging cell lines following incubation with predatory bacteria:

To further demonstrate that predatory bacteria have no negative effect on mammalian cell viability and structure, HaCAT human epidermal keratinocytes and Hs27 human skin fibroblasts were exposed for 4 and 24 hrs to predatory bacteria ($1x10^9$). Thereafter, light microscopy and Fluorescent microscopy were used to evaluate cell morphology. In these experiments PBS was used as a negative control while Triton X and *P. aeruginosa* Pa14 were used as a positive control.

Result and conclusion. As seen in the figure below, total cell detachment was seen when the examined cell lines were exposed for 4 hrs or more to the Triton or *P. aeruginosa* Pa14. However, no cell detachment or morphological changes were seen in cells which were incubated with the predators. Conforming that predatory bacteria are non toxic to mammalian cell lines.

	HaCAT		HS27	
	4hr exposure	24hr exposure	4hr exposure	24hr exposure
PBS				
Triton X				
Pa14				
Bb 109J				
Bb HD100				
Mica				

Subtask 2.2. *Determine the influence of predator exposure on cell cytokine profile.*

Rationale. Exposing cells to microbial challenge could alter cell physiology. Among the changes less desired in wounds, which are already challenged by bacteria, is the overproduction of proinflammatory immune mediators. To examine the effect of predatory bacteria on cell cytokine profile, HaCAT and Hs27 cell lines were grown in 24 well plates and exposed for 4 hrs to 10^9 PFU/ml of each of the 3 predators. Thereafter, the culture media was collected and the pro-inflammatory cytokine profile was measured using the Human High Sensitivity Cytokine Panel (Millipore). Non-bacteria PBS (Mock) exposed cells were used as negative control. For positive control, cells will be exposed to *P. aeruginosa* PAO1 which is known to be less cytotoxic than the PA14 strain, thus allowing positive cytokine induction while limiting cell death. Each experiment was conducted with 4 wells for each treatment.

Cytokine profile of HaCAT cells following incubation with predatory bacteria.

IL-2 - HaCAT

IL-6 - HaCAT

IL-4 - HaCAT

IL-7 - HaCAT

IL-5 - HaCAT

IL-8 - HaCAT

TNF-A - HaCAT
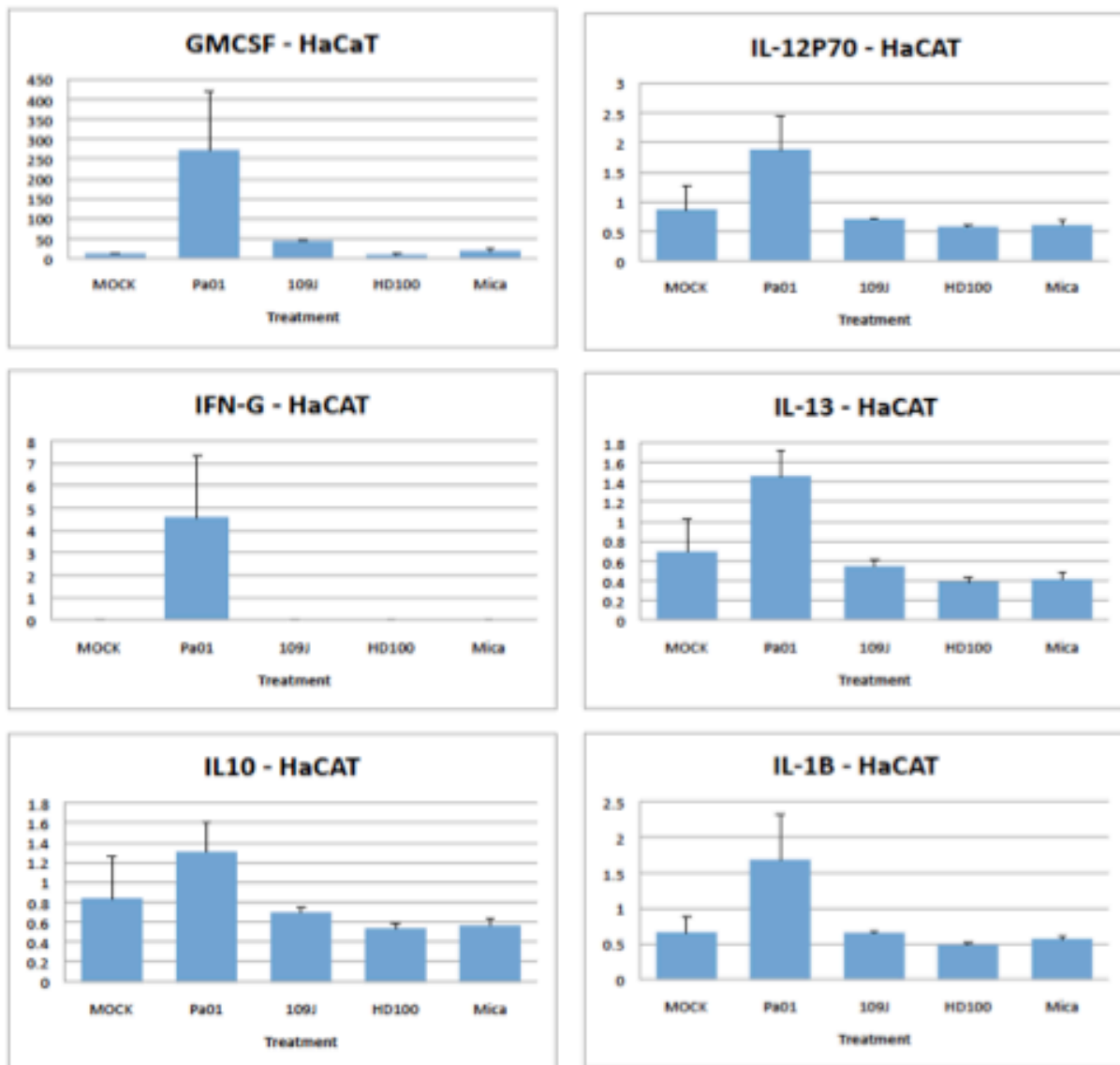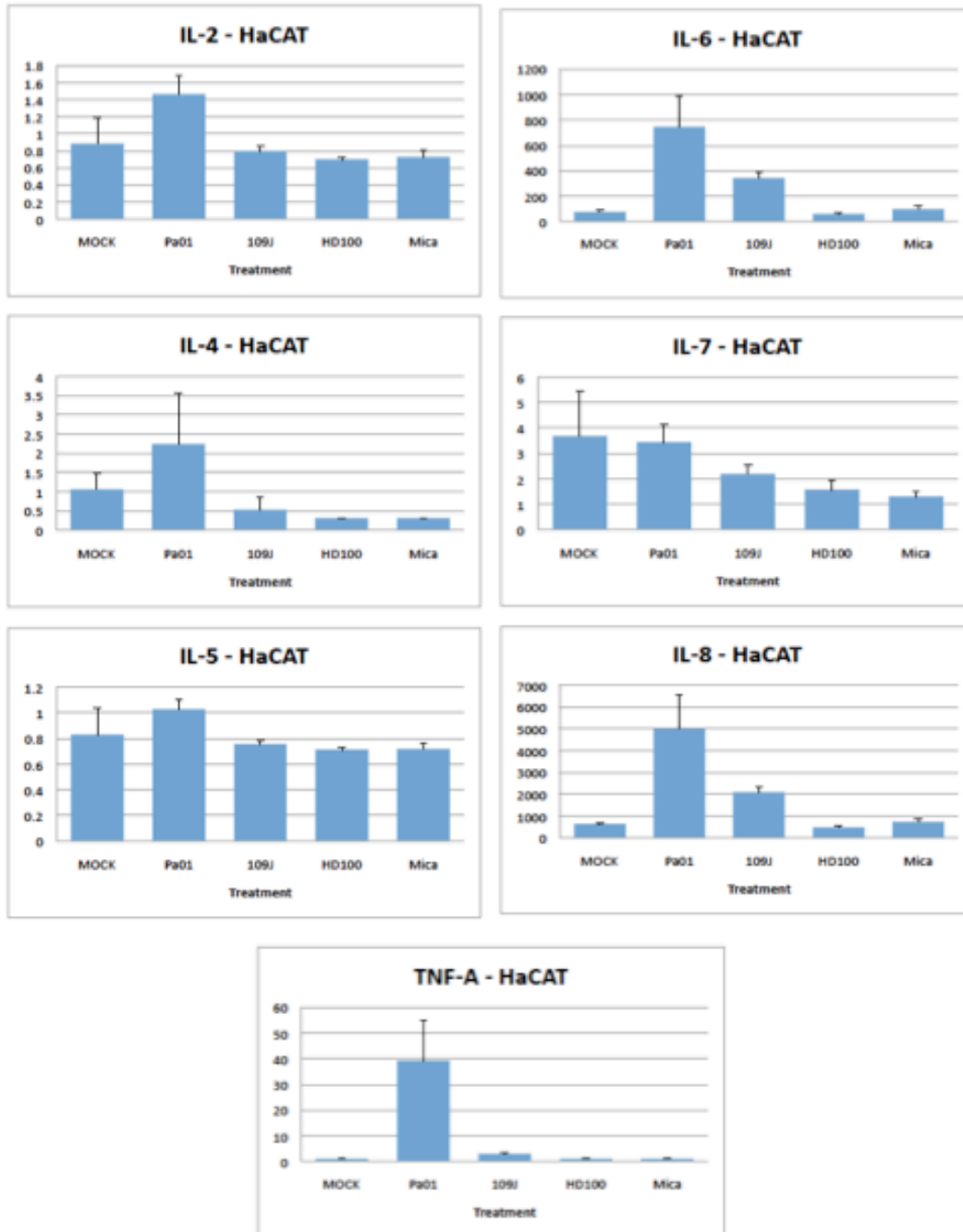
Results. Our data shows that exposing HaCAT cells to high levels of predatory bacteria causes no elevation in cytokine production in 11 out of the 13 examined cytokines. However, both IL-8 and IL-6 were slightly elevated, compared to the control, in cells exposed to *Bdellovibrio* 109J. An increase in cytokine production was seen in 11 out of the 13 examined cytokines after exposure to *P. aeruginosa* PAO1. For IL-5 and IL-7, compatible cytokines levels were measured for the control and *P. aeruginosa* treated sample.

Cytokine profile of Hs27 cells following incubation with predatory bacteria.
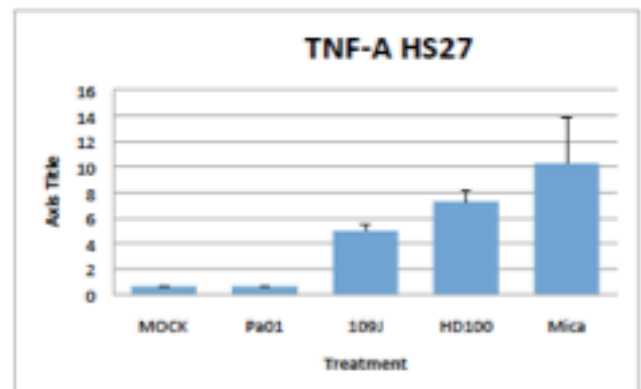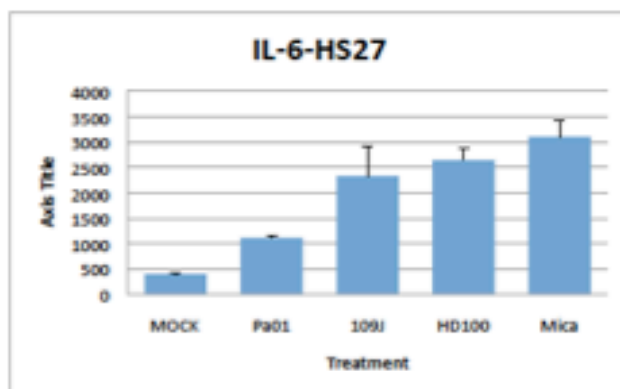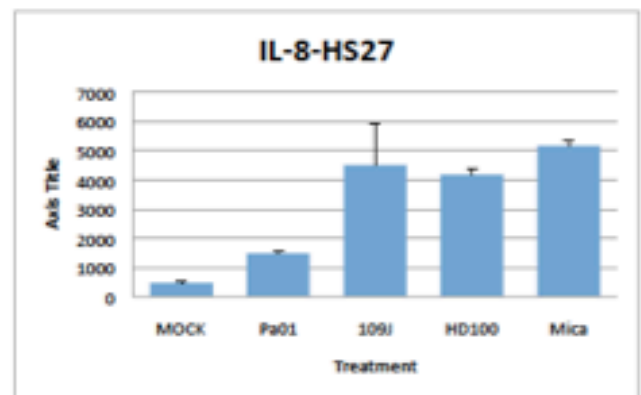
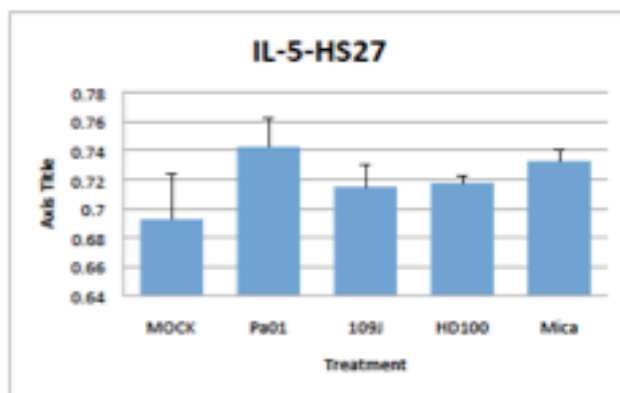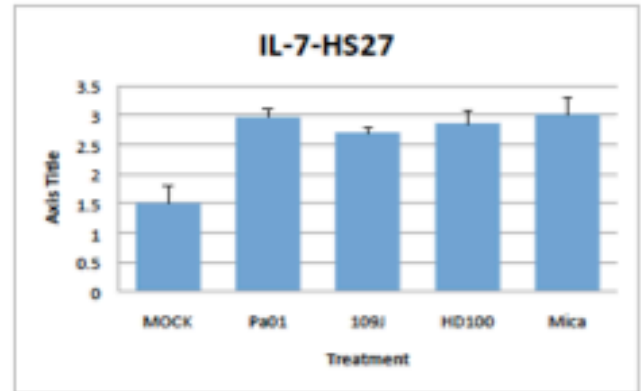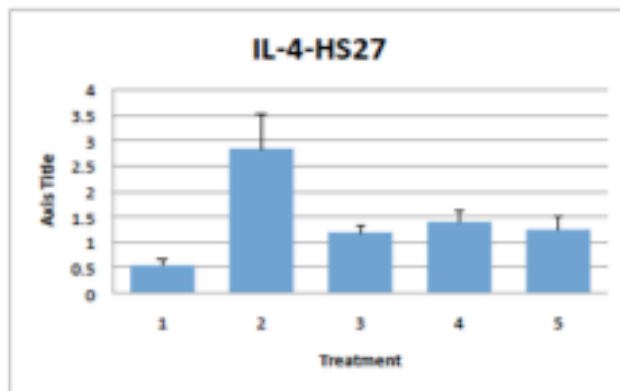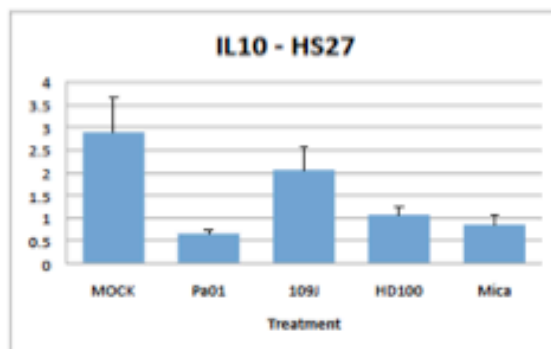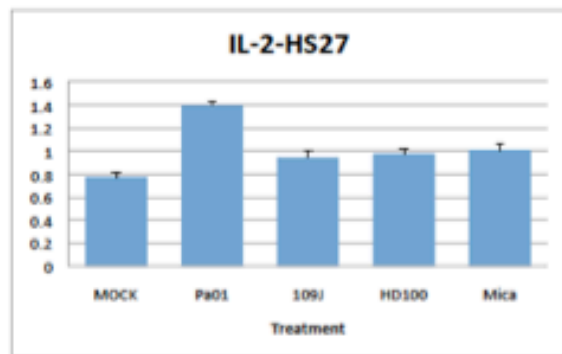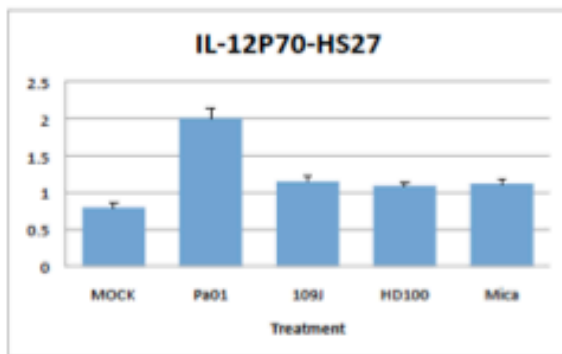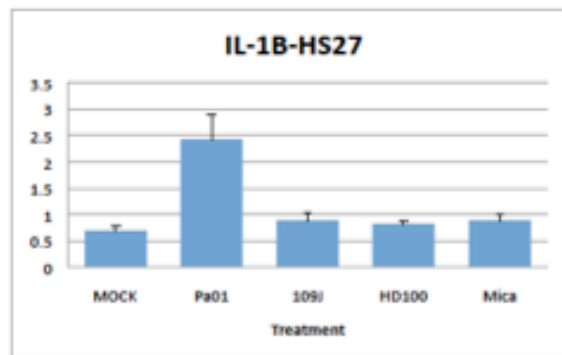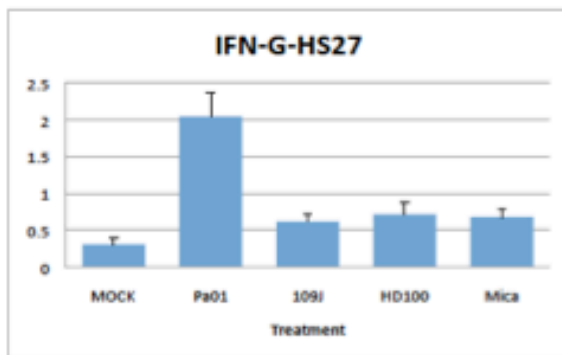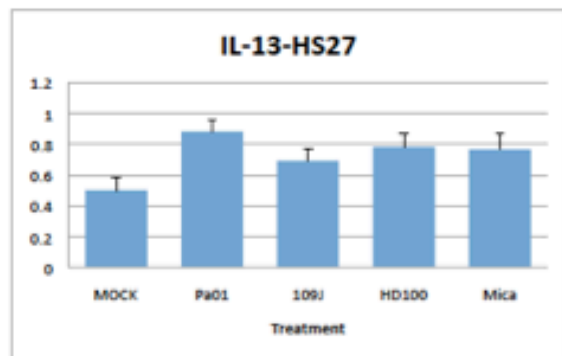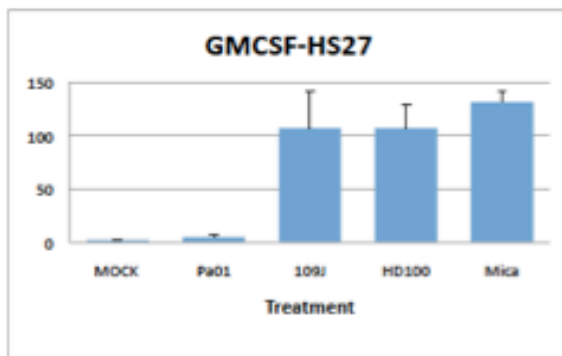**Results.** The cytokine profile of Hs27 cells exposed to predatory bacteria was found to be different than the HaCAT cells. Three of the 13 cytokines (IL-2, IL-12 and IL-1β) showed no change compared to the PBS control. IL-10 (an anti-inflammatory cytokine) was found to be reduced in cells exposed to the bacteria compared to the PBS control (Mock). Four out of the 13 cytokines were slightly elevated, compared to the PBS control, but less than the *P. aeruginosa*. IL-7 expression was found to be stimulated to similar levels by all bacteria treatments. Finally, exposing the cells to predatory bacteria had caused elevation in IL-6, IL-8, TNF-A and Granulocyte-macrophage colony-stimulating factor (GM-CSF).

Aim III. Efficacy and toxicity in animal models.

Subtask 3.1. *Determining the toxicity/efficacy of predatory bacteria in a mouse puncture wound model.*

The goal of this aim was to evaluate whether predatory bacteria have a toxic effect on small animals using a range of doses as inoculum into open wounds. In this task we had used a murine wound model of infection developed by collaborators at WRAIR (Zurawski Lab). This part of the study was done and reported by Dr. Zurawski at WRAIR.

Objective: The primary objective of these experiments is to assess the safety of live *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments are weight loss / gain over time of the mice, mice clinical score (recorded daily), Histopathology report on wound samples taken 24h post-surgery, Cytokine profile, and mice wound size. These experiments use either cyclophosphamide treated mice (immunocompromised model) or normal mice. Treatment was done with a single application of predatory bacteria in PBS.

Questions to be answered

Safety of *Bdellovibrio bacteriovorus (*Bb) or *Micavibrio aeruginosavorus (MA)* application and dose effect on immunocompromised mouse health.

Time-to-close – Is the kinetic of wound closure in this immunocompromised model changes upon treatment with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus*?

Clinical signs of wounding – Is there a change in clinical scores upon treatment with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus*?

Weights – Is the weight of mice treated with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* altered compared to mock treated mice?

Gross pathology – Does gross wound appearance of mice change with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* application?

Histopathology of wound healing post wound closure – Does the wound appear in any way different from histopathology perspective upon treatment with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus*?

Does exposure of the wound to *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* affects the cytokine profile in the mouse once the wound closes?

Experiment 1.

Objective. The primary objective of the experiment was to assess the safety of live *Bdellovibrio bacteriovorus* administration on exposed wounds in the mouse wound model.

The primary measures of safety in these experiments were weight loss of mice and mice clinical score taken daily.

The experimental groups were:

- G1 - 5 mice wounded and treated once with $5x10^8$ PFU/wound *Bdellovibrio bacteriovorus 109J.*

- G2 - 5 mice wounded and treated once with $5x10^4$ PFU/wound *Bdellovibrio bacteriovorus 109J.*

- G3 - 5 mice wounded and treated once with $5x10^8$ PFU/wound *Bdellovibrio bacteriovorus HD100.*

- G4 - 5 mice wounded and treated once with $5x10^4$ PFU/wound *Bdellovibrio bacteriovorus HD100.*

- G5 – 4 Mice wounded and treated with PBS to serve as controls for treatments

Time points and samples collected:
Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).

Outcome.
No Significant difference with respect to weight and or wound size was detected.

Experiment 2.

Objective: The objective of this experiment is to assess the safety of live *Bdellovibrio bacteriovorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments were weight loss of mice and mice clinical score taken daily. This experiment was a biological repeat and includes 9 additional mice used for the 24h post infection time point cytokine and histological analysis.

The experimental groups were:

- G1 – 5+3 mice wounded and treated once with $2.5x10^8$ PFU/wound *Bdellovibrio bacteriovorus 109J.*

- G2 - 5 mice wounded and treated once with $2.5x10^4$ PFU/wound *Bdellovibrio bacteriovorus 109J.*

- G3 – 5+3 mice wounded and treated once with $1.62x10^8$ PFU/wound *Bdellovibrio bacteriovorus HD100.*

- G4 - 5 mice wounded and treated once with 1.62×10^4 PFU/wound *Bdellovibrio bacteriovorus HD100*.

- G5 – 5+3 Mice wounded and treated with PBS to serve as controls for treatments

Time points and samples collected: Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).
Three mice from groups G1, G3, and G5 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Outcome.
No Significant difference with respect to weight and or wound size was detected. No difference in the histology analysis at 24h post-surgery was detected.

Experiment 3.

Objective: The primary objective of this experiment is to assess the safety of live *Bdellovibrio bacteriovorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments will be weight loss of mice and mice clinical score (recorded daily). This experiment uses cyclophosphamide (CP) treated mice (immune-compromised model) to assess safety.

The experimental groups were:

- G1 – 5+3 CP pre-treated mice wounded and treated once with 5×10^8 PFU/wound *Bdellovibrio bacteriovorus 109J*.

- G2 - 5 CP pre-treated mice wounded and treated once with 5×10^4 PFU/wound *Bdellovibrio bacteriovorus 109J*.

- G3 – 5+3 CP pre-treated mice wounded and treated once with 8×10^8 PFU/wound *Bdellovibrio bacteriovorus HD100*.

- G4 - 5 CP pre-treated mice wounded and treated once with 8×10^4 PFU/wound *Bdellovibrio bacteriovorus HD100*.

- G5 – 5+3 CP pre-treated mice wounded and treated with PBS to serve as controls for treatments.

Time points and samples collected:
Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).

Three mice from groups G1, G3, and G5 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Outcome.
No Significant difference with respect to weight and or wound size was detected.
No difference in the histology analysis at 24h post-surgery was detected.

Experiment 4.

Objective: The primary objective of this experiment is to assess the safety of live *Micavibrio aeruginosavorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments will be weight loss of mice and mice clinical score (recorded daily). This experiment uses cyclophosphamide (CP) treated mice (immune-compromised model) to assess safety.

The experimental groups were:

- G1 – 5+3 mice wounded and treated once with $1x10^8$ PFU/wound *M. aeruginosavorus*.

- G2 – 5+3 mice wounded and treated once with $1x10^4$ PFU/wound *M. aeruginosavorus*.

- G3 – 5+3 Mice wounded and treated with PBS to serve as controls for treatments

- G4 – 5+3 CP pre-treated mice wounded and treated once with $5x10^8$ PFU/wound *M. aeruginosavorus*.

- G5 – 5+3 CP pre-treated mice wounded and treated once with $5x10^4$ PFU/wound *M. aeruginosavorus*.

- G6 – 5+3=8 CP pre-treated mice wounded and treated with PBS to serve as controls for treatments.

G1, G3-G6 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Time points and samples collected:
Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).
Three mice from groups G1, G3-G6 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Outcome.
No Significant difference with respect to weight and or wound size was detected.
No difference in the histology analysis at 24h post-surgery was detected.

Conclusion. Predatory bacteria do not seem to have a negative effect on mice wellbeing or wound healing when administered to open wounds of healthy animals or animals which are immunocompromised.

Average data collected throughout the aim study was analyses summarized and reported below.

Results

All mice survived the treatment and were on course to full recovery or fully recovered by the end of the experiment.

Clinical scores were similar in all groups during the first days until no clinical signs could be observed.

Weight loss trends from day (-4 or 0) to the last day that measurements were taken post operation are similar among the groups. Treatment with high dose of HD100 seems to result in a transient increased weight loss that is most obvious at day 2.

There seems to be no difference between treatment and control with respect to the effect on weight later in the time course.

There seems to be little to no effect of CP treatment on the weight and no real difference among the groups except for the CP treated 10^4 applied *Micavibrio aeruginosavorus*. We assume this is as a result in the variance between the animal and the specific execution of that experiment.
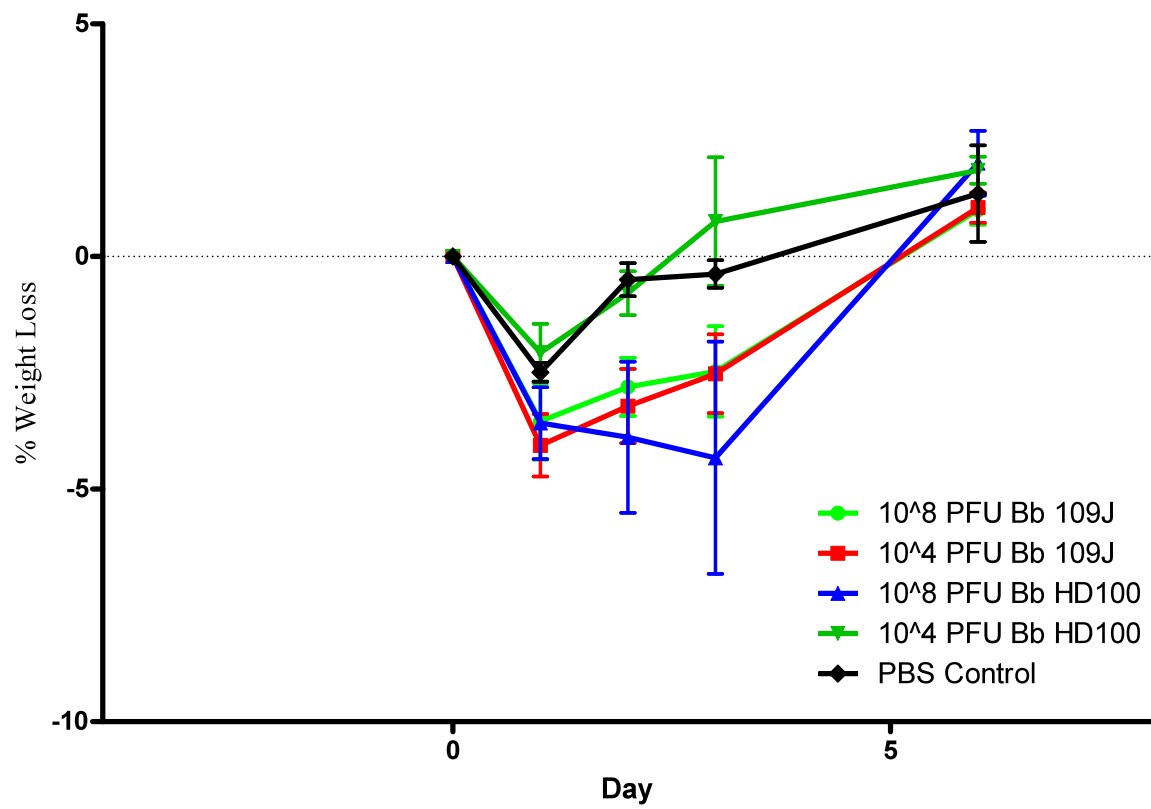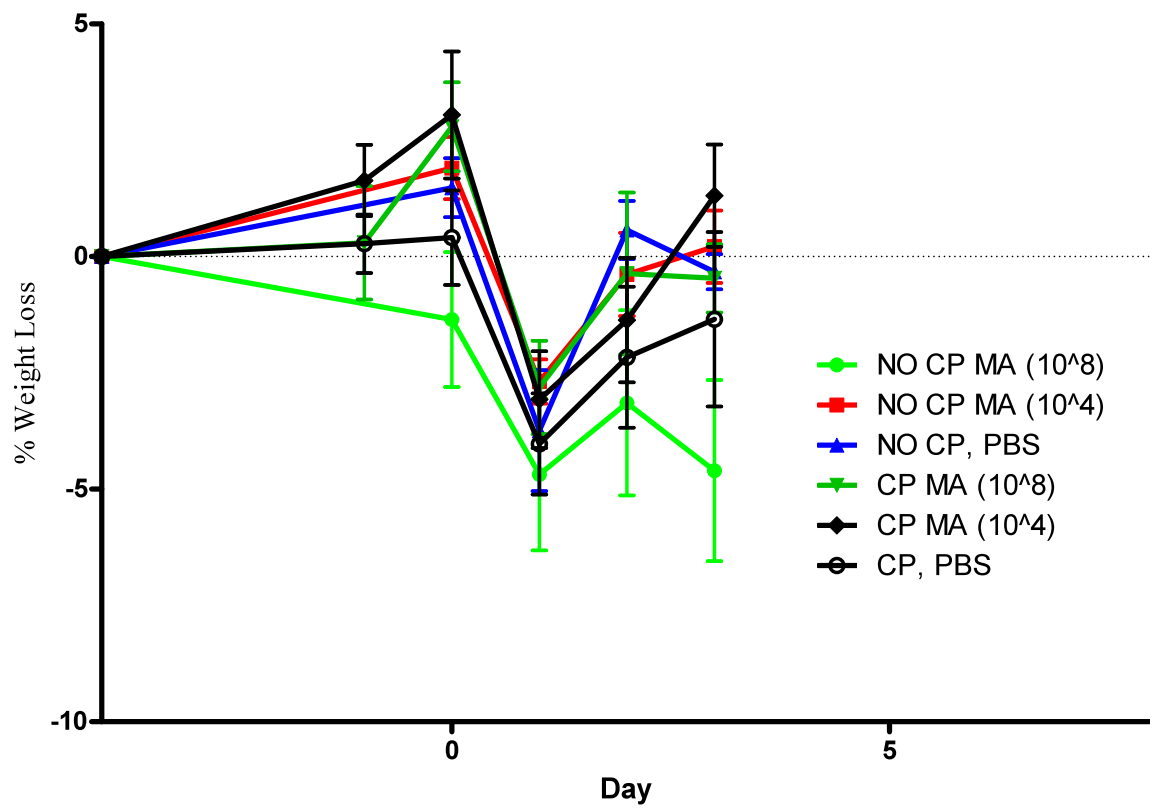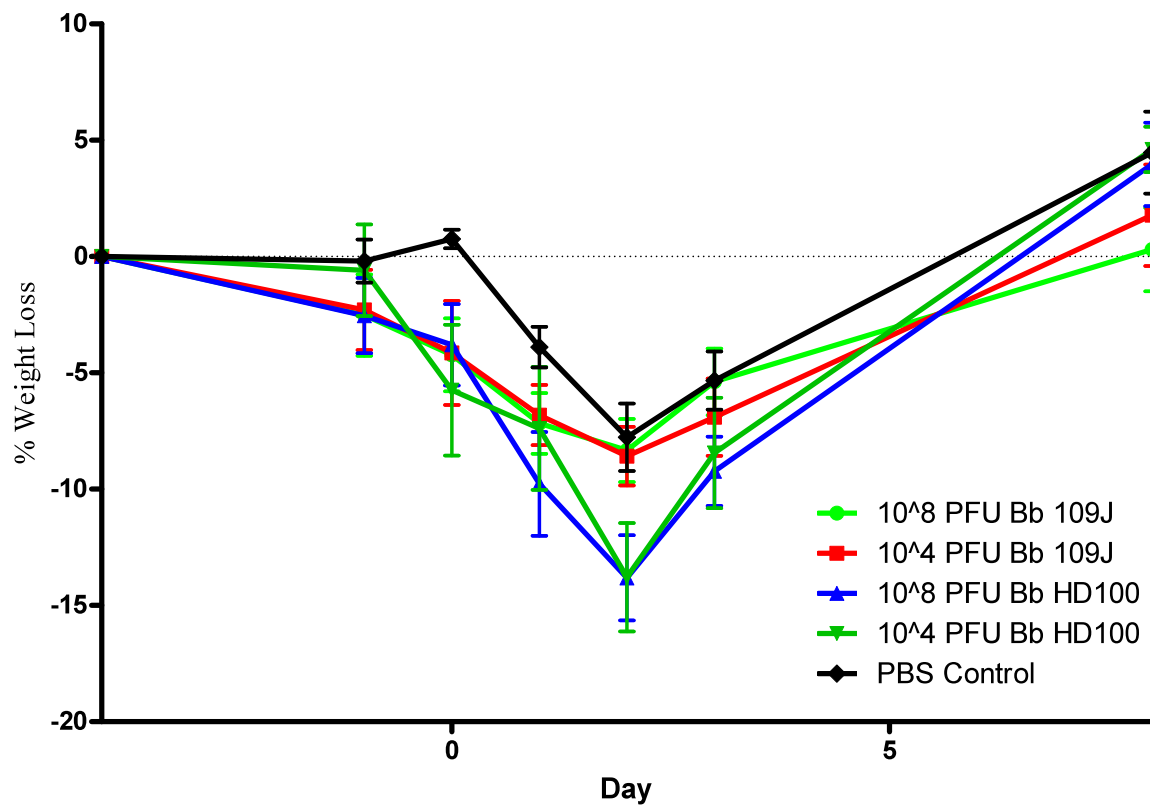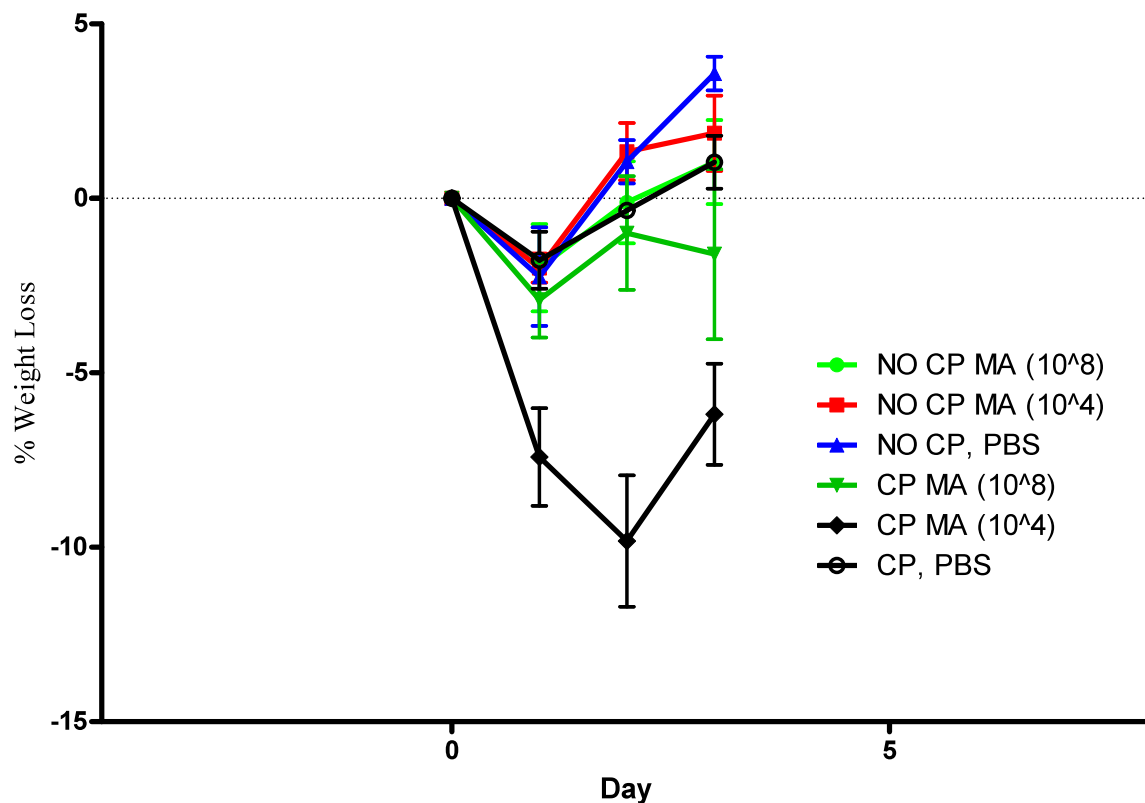
Figure 1 – Percentage weight loss in groups 1-5 over 12 days. Day (-4) & Day (-1) CP treatment, Day 0 – surgery, Day 0 treatment. Data for euthanized mice (if any) is not included.

The different treatments were almost identical in the time required for the wound to completely close. However, differences in the healing process between the Non-CP groups treated with *Micavibrio aeruginosavorus* and the rest of the groups in that experiment can be seen – this is a favorable trend and thus we see it as a positive indicator. Other minor differences in the time to close between treated with Bd and the control PBS treated group can be observed in Figure 2.
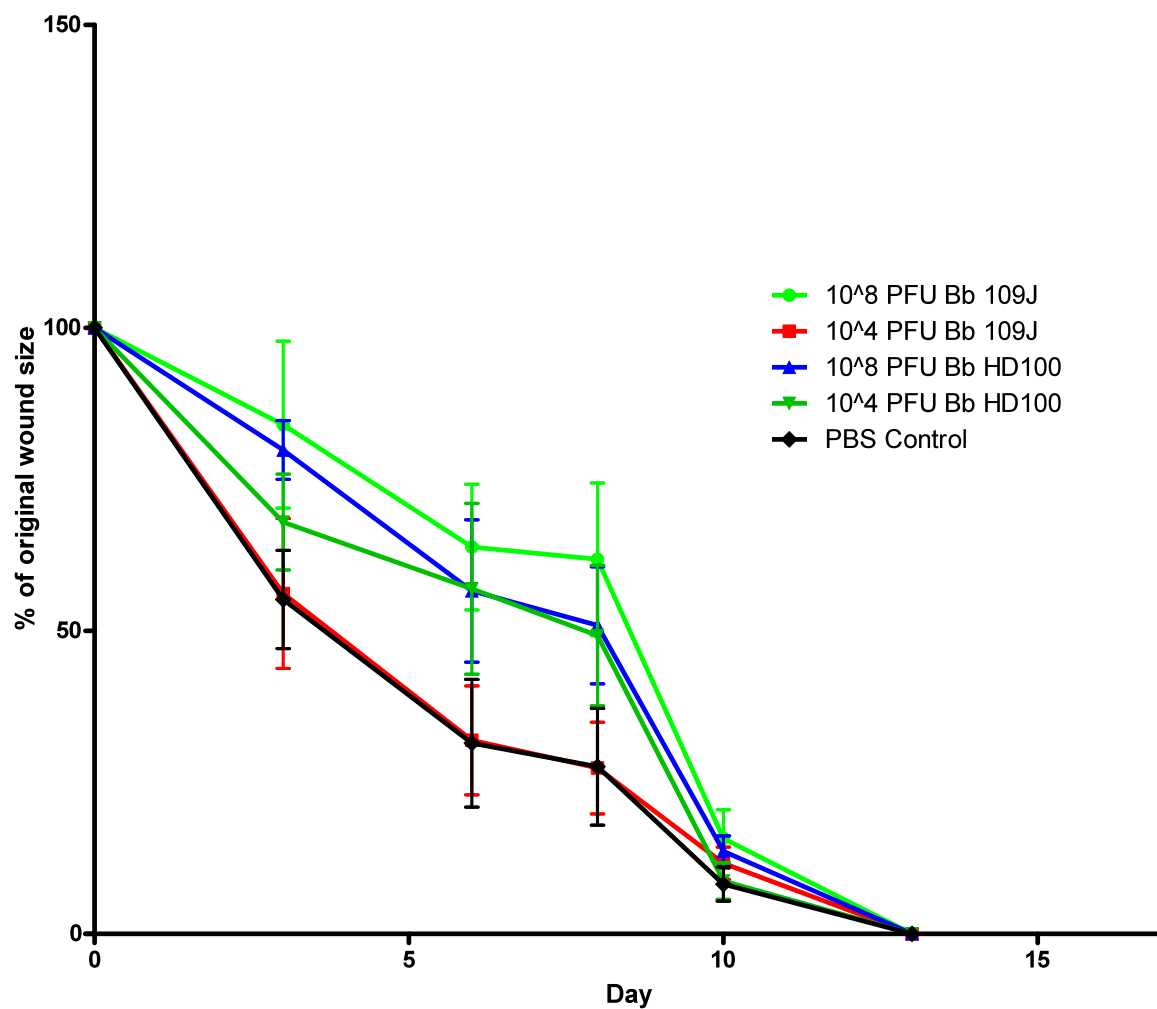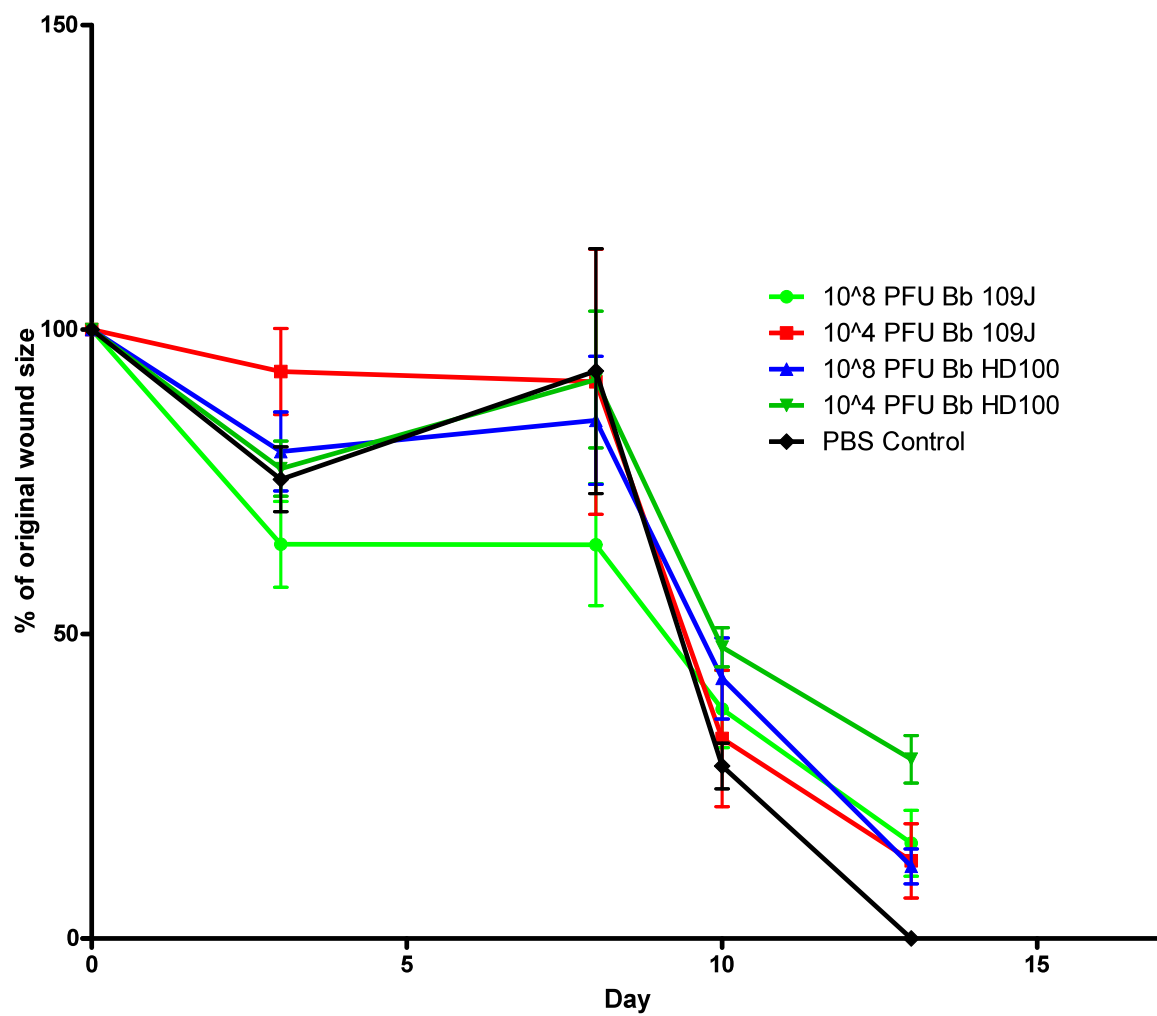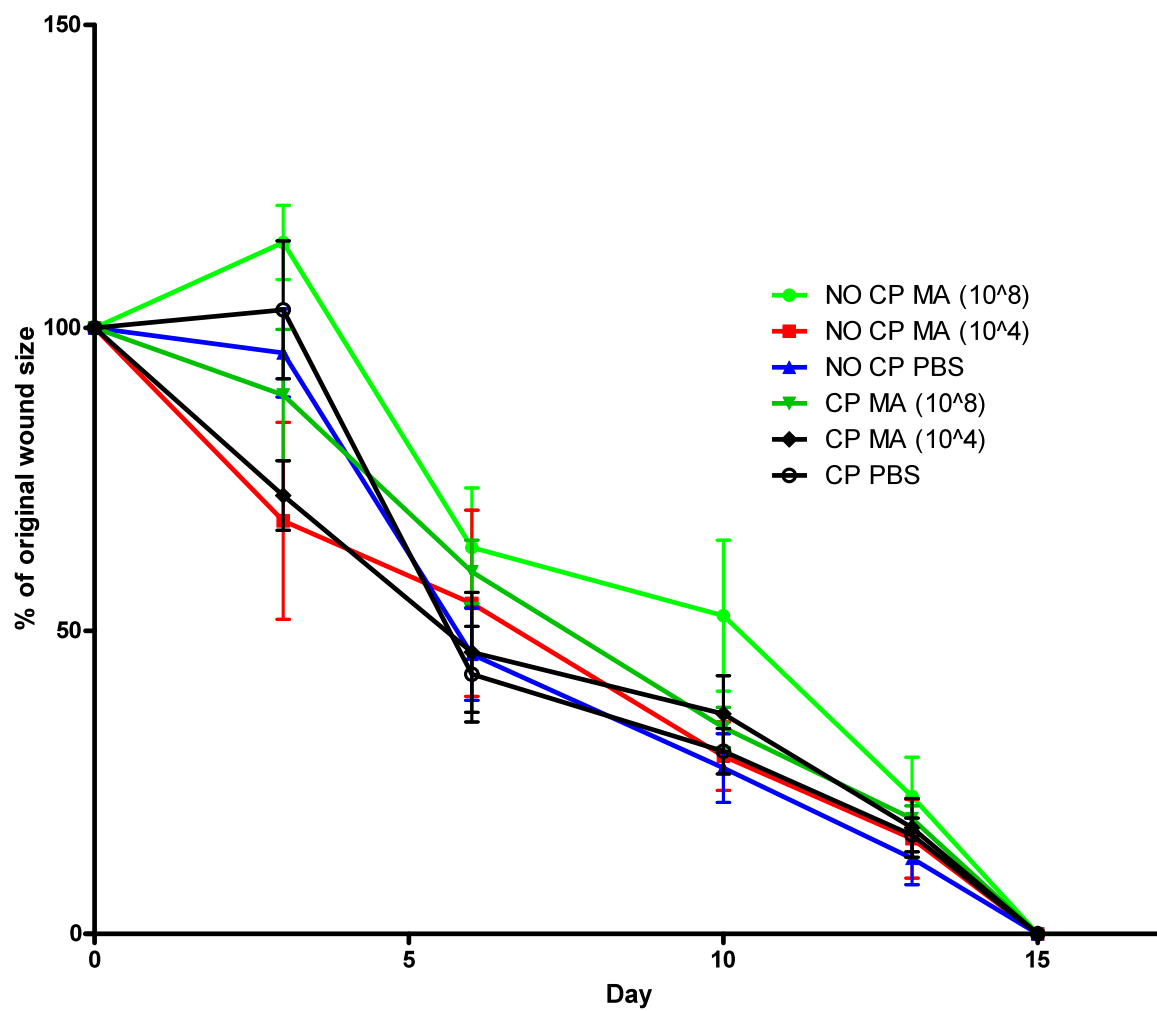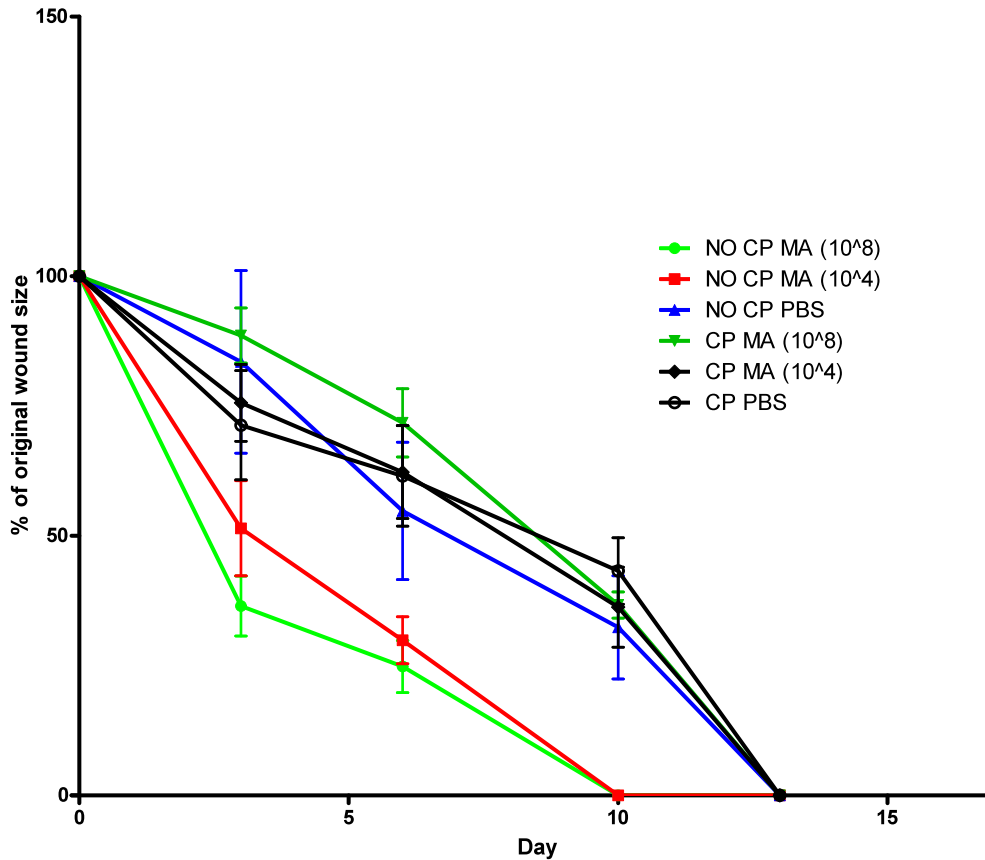
Figure 2 – Percentage of wound size in groups 1-5 over 17 days. Data for euthanized mice (if any) is not included.

Conclusions.

Predatory bacteria are safe when applied topically to open wounds of CP or non CP treated mice (only MA was examined in non-CP treated mice) with doses ranging up to 10^8 PFU causing, if anything, minor effects on weight and a possible positive effect on time it takes the wound to heal. We recommend using the J109 strain as it did not deviate from the PBS control as much as the deviations of the HD100 strain.

Predatory bacteria are non pathogenic in a mice wound model.
Administering high doses (1x10⁸) of *B. bacteriovorus* 109J, HD100 and *M. aeruginosavorus* to open wounds of healthy animals and animals who are immunocompromised did not have a negative effect on mice well-being or wound healing.

| Initial wound | PBS control | *B. bacteriovorus* 109J | *B. bacteriovorus* HD100 | *M. aeruginosavorus* | *K. pneumoniae* |
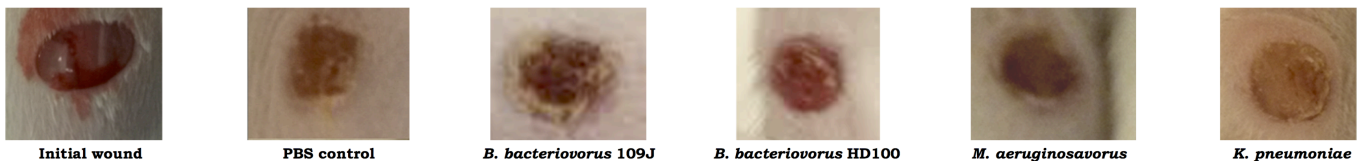
Wound closure 8 days following inoculation with predatory bacteria or control.

PATHOLOGY REPORT

Walter Reed Army Institute of Research, Silver Spring, MD
Naval Medical Research Center, Silver Spring, MD

Accession Number	Animal ID	Protocol Number		Investigator	Pathologist
14-1484	FY1412880	14-BRD-01S		ZURAWSKI	CLH
Species	Strain	Sex	Weight	Room	Method of Euthanasia
MOUSE	BALB/C	FEMALE	<50 G	GW171	KET/XYL OVERDOSE
Date Received	Date Necropsied	Prosector		Type of Necropsy	Report Date
5/27/2014	5/27/2014	HONNOLD		TRIM IN	6/24/2014

History

A 6mm excisional wound was created, mice wounded and treated once with 5.0X10^8 PFU Micavibrio aeruginosavorus.

Macroscopic Findings

Only skin sample with wound submitted for evaluation.

This report will serve as a final report for the following consecutive accession numbers: 14-1484 through 14-1501.

Microscopic Findings

The following morphologic fits for all mice identified as 'Day 2': Haired skin, site of experimental wound: Dermatitis, acute (neutrophilic), focally extensive, marked to severe, with dermal edema, serocellular exudate and crusting.

Comments

Histologic evaluation of the day 2 wounds did not reveal any differences between groups. The experimental lesion, from a histologic standpoint, is consistent with typical wounding. The predominant inflammatory response is neutrophilic. Unfortunately, I was unable by light microscopy to appreciate any discernible difference between the groups.

Cary Honnold, DVM, Diplomate ACVP, ACVPM

Pathology Report, 14-brd-01S, page 1 of 1

Pathologist Signature: CARY.HONNOLD
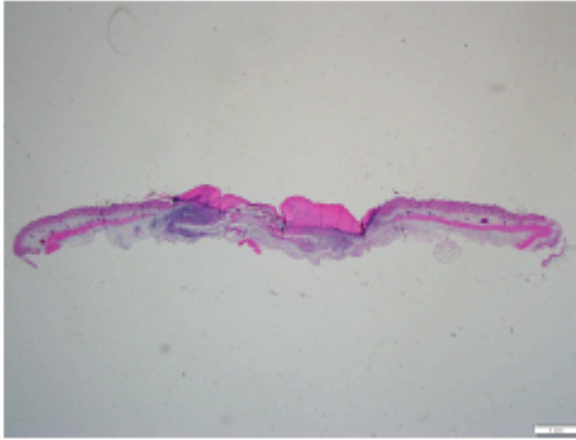
Haired skin from 14-1485, 2 day wound, 1B2, Non CP treated.
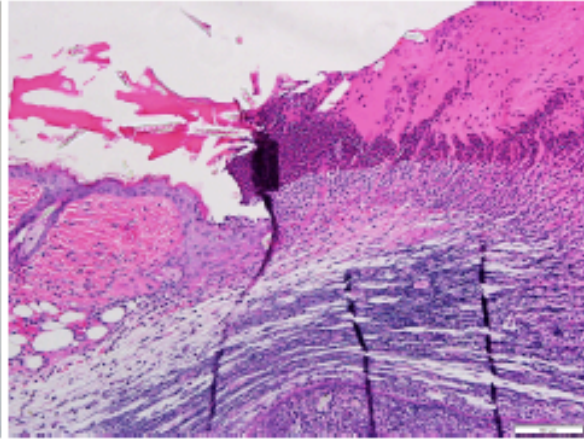
Haired skin from 14-1485 demonstrating acute inflammation at wound edge. Typical for Day 2 wounds.
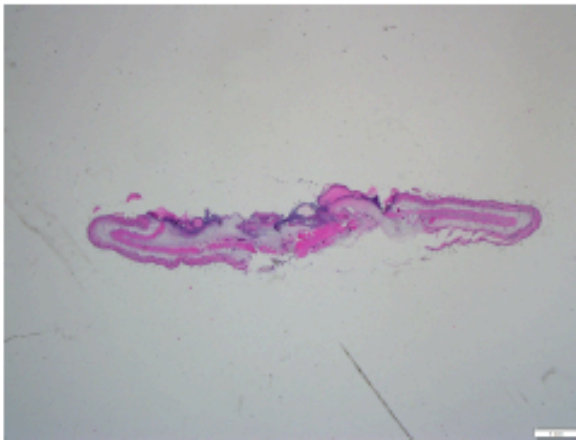
Wound from PBS treated.
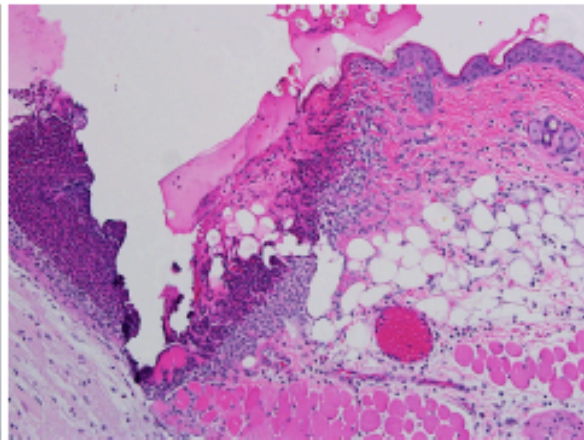
Wound edge from PBS treated.

PATHOLOGY REPORT

Walter Reed Army Institute of Research, Silver Spring, MD
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ACCESSION NUMBER: 14-0541	ANIMAL ID: FY132472

ACCESSION 14-0541	CONTRIBUTOR ZURAWSKI	DIVISION/DEPARTMENT BRD-WI				DATE OF REPORT 13-Dec-13	
PROTOCOL 14-BRD-01S	**TYPE** TRIM IN	**DATE SUBMITTED** 27-Nov-13				**DATE COMPLETED** 13-Dec-13	
SPECIES MOUSE	**STRAIN** BALB/C	**AGE** 6-12WKS	**SEX** F	**WEIGHT** <40 LBS		**ANIMAL ID** FY132472	**ROOM NO** GW173

HISTORY

TREATED 109J, A 6MM EXCISIONAL WOUND WAS CREATED, MICE WOUNDED AND TREATED ONCE WITH 10^8 PFU. BDELLOVIRIO BACTERIOVIRUS 109J

This report is for the following pathology accession numbers, animal numbers, and tube number reference:
1. 14-0541/FY132472/109J
2. 14-0542/FY132474/109J
3. 14-0543/FY132474/109J
4. 14-0544/FY132475/HD100
5. 14-0545/FY132476/HD100
6. 14-0546/FY132477/HD100
7. 14-0547/FY132478/HD100
8. 14-0548/FY132479/Control PBS
9. 14-0549/FY132480/Control PBS

MACROSCOPIC FINDINGS

The submitted skin samples were from the back of the mouse and contained a focal wound with overlying crusting.

MICROSCOPIC FINDINGS

The following morphological diagnosis is essentially representative for all 9 skin wounds examined.
1. Haired skin: Dermatitis, ulcerative, neutrophilic (acute), focal, severe, with edema, serocellular exudate, and underlying neutrophilic myositis.

COMMENTS

The 9 submitted skin samples were examined histologically and there was no discernable difference between control and treated groups. Histologically, the wounds consist of a robust, acute, neutrophilic inflammation, marked edema in surrounding tissue, and serocellular exudate. The neutrophils are also present within the underlying skeletal muscle.

Cary Honnold, DVM, Diplomate ACVP, ACVPM

Key Research Accomplishments and Results.

Aim-1, Subtask 1.1. Development of genetically stable resistance to predation.

- Our data suggest that no genetically stable predation resistant phenotype developed in *K. pneumoniae* and *A. baumannii* following sequential predation by *B. bacteriovorus* 109J and *B. bacteriovorus* HD100.

- Our data suggest that no genetically stable predation resistant phenotype developed in *P. aeruginosa* Pa14 following sequential predation by *M. aeruginosavorus*.

- Our data suggest that host cells grown as a biofilm do not adapt to form predation resistant biofilms.

- *K. pneumoniae* ATCC 33495 prey cells which were randomly mutagenized by transposon insertion or UV radiation yield no stable resistance to predation by *B. bacteriovorus* 109J or *M. aeruginosavorus*.

Aim-1, Subtask 1.2. Investigating the ability of the predator to breach its host specificity and attack previously resistant bacteria.

- Our data suggest that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This finding was verified using both Gram-negative and Gram-positive host.

- Our data suggest that *M. aeruginosavorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This finding was verified using both Gram-negative and Gram-positive host.

Subtask 1.3. Enriching for hyper predatory variants.

- Our data suggest that *B. bacteriovorus* 109J could become more virulent on a particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria.

- Our data shows that after continuous predation cycles *M. aeruginosavorus* does not seem to develop an ability to become hyper virulent on *A. lwoffii*.

- Our data suggest that *Bdellovibrio* 109J and *Micavibrio* could be acclimated to prey at an elevated temperature of 39°C.

Aim II. Determining the effect of predatory bacteria on eukaryotic cells.

Subtask 2.1. *Determining the toxicity of predatory bacteria on mammalian cells.*

- Our data suggest that *B. bacteriovorus* 109J, HD100 and *Micavibrio* are non toxic to HaCAT human epidermal keratinocytes, Hs27 human skin fibroblasts and L929 murine fibroblasts and do not cause reduction in cell viability.

- Microscopic imaging of HaCAT and Hs27 cells exposed to the predators had revealed no change in cells cell morphology or detachment.

Subtask 2.2. *Determine the influence of predator exposure on cell cytokine profile.*

- Our data show that exposing HaCAT cells to high levels of predatory bacteria had caused no elevation in cytokine production in 11 out of the 13 examined cytokines. However, exposing Hs27 human skin fibroblasts to the predators did result in an increase in cytokines production.

Aim III. Efficacy and toxicity in animal models.

Subtask 3.1. *Determining the toxicity/efficacy of predatory bacteria in a mouse puncture wound model.*

The effect of applying predatory bacteria to open wounds was evaluated in both naïve and immunocompromised mice. Experiments were conduct using *B. bacteriovorus* 109J, HD100 and *M. aeruginosavorus*. Our findings show that when applied to open wound, predatory bacteria do not seem to cause any adverse effect to the animal. No significant difference with respect to weight loss, wound clinical score and wound time to close was detected in wound "infected" with the predator. No difference in the histology analysis was seen in mice inoculated with the predators.

Conclusions. Predatory bacteria are safe when applied topically to open wounds of naïve and immunocompromised mice.

Reportable Outcome

Manuscripts.

During the lifetime of this proposal we have published two manuscripts and a review. The manuscripts discuss the ability of predatory bacteria to attack defined drug resistant pathogens as well as to treat pathogens associated with eye infection. Although **no** specific funds from this grant were used in the research leading to the publications, some of the big item equipment purchased through this grant was utilized. The work is also within the scope of our long-term objective of using predator bacteria to treat human infection.

1. **Kadouri, E. D.,** To, K., Shanks, M. Q., and Doi, Y. 2013. Predatory Bacteria; A Potential Ally against Multidrug-Resistant Gram-Negative Pathogens. PLoS ONE. 8(5): e63397. doi:10.1371/journal.pone.0063397.

2. Shanks, M. Q., Davra, R. V., Romanowski, G. E., Brothers, M. K., Stella, A. N., Godboley, D., and **Kadouri. E. D.** 2013. An Eye to a Kill: Using Predatory Bacteria to Control Gram-Negative Pathogens Associated With Ocular Infections. PLoS ONE. PLoS ONE 8(6): e66723. doi:10.1371/journal.pone.0066723.

3. Shanks, R. M., and **Kadouri E. D.** 2014. Predatory prokaryotes wage war against eye infections. Future Microbiology. 9:429-432.

Oral Presentations.

Several of the findings supported by this grant were presented by the PI at the following invited Presentations. (No abstracts were submitted).

1. **Kadouri, E. D.** Controlling Drug Resistant Bacteria- The Answer is Out There. Department of Oral Biology seminar series. New Jersey Medical School. Newark, NJ. February. 2013.

2. **Kadouri, E. D.** Controlling Drug Resistant Bacteria- The Answer is Out There. Department of Biochemistry, Microbiology and Immunology. University of Ottawa, Faculty of Medicine. Ontario, Canada. April. 2013.

3. **Kadouri, E. D**. Bio-control of Drug Resistant Bacteria. Physiology, Ecology and Taxonomy (NEMPET) Meeting. Blue Mountain Lake, NY. June 2013.

4. **Kadouri, E. D**. The use of predatory bacteria to control select pathogens and treat respiratory infections, Grant kickoff meeting, DoD, Defense Advanced Research Project Agency (DARPA), Virginia. January 2014.

Poster Presentations.

1. **Kadouri, E. D.,** and Godboley, D. The use of predatory prokaryotes to control human pathogens and biofilms. 4th ASM Conference on Beneficial Microbes. San Antonio, Texas, October 2012.

2. Davra, R., Mukherjee, S., Gancz, H., Zurawski, D., and **Kadouri, E. D.** The use of predatory bacteria to control select pathogens and treat respiratory infections. Military Health System Research Symposium (MHSRS). Fort Lauderdale, FL. August 2012.

Student research opportunities at Rutgers.

This project had given a few students the opportunity to conduct research and gather hands-on scientific experience.

<u>**Research Assistant**</u>
Somdatta Mukherjee.
Shilpi Gupta.

<u>**Rotation Student**</u>
Andrew Kim-Rotation M. S. student (GSBS-Rutgers).
Ameet Patheja- Rotation M. S. student (GSBS-Rutgers).
Vanessa Sahs- Rotation M. S. student (GSBS-Rutgers).
Gregory Ramirez- M.S. candidate (GSBS-Rutgers).
Kenneth Shatzkes- Ph. D candidate (GSBS-Rutgers).
Chi Tang- Rotation M. S. student (GSBS-Rutgers).
Fernanda Morais- Rotation M. S. student (GSBS-Rutgers).

Additional funding.
Information generated via this grant was used as preliminary data to secure the following grant:

ARO Agreement W911NF-14-2-0016
DoD, Defense Advanced Research Project Agency (DARPA)
The use of predatory bacteria to control select pathogens and treat respiratory infections.
$580,516   12/2013- 12/2014

Conclusion

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, with many traditional antimicrobial agents becoming ineffective. Our main hypothesis is that predatory prokaryotes could serve as a novel therapeutic agent to control wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to prey on a wide range of pathogens including bacteria isolated from Wounded Warriors. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria and investigating predator prey interactions and resistance.

It was proposed that, unlike antibiotics or phage therapy, the selective pressure of predation does not generate genetically stable resistant variants in the host. In order to evaluate this hypothesis we conduct experiments aimed at increasing the selective pressure on the host and assessing if any genetically stable predation resistant phenotypes emerge. Using enrichment culturing techniques we have verified that no genetically stable predation resistant phenotype developed in *K. pneumoniae* and *A. baumannii* host cells following sequential predation by *B. bacteriovorus* 109J or HD100. Furthermore, sequential predation by *M. aeruginosavorus* also did not yield resistance in *P. aeruginosa.* As was seen with liquid cultures, *A. baumannii* host cells grown as a biofilm do not adapt to form predation resistant biofilms as a consequence of predation by *B. bacteriovorus* 109J or HD100.

We have previously conducted experiments aimed at investigating the host range of each predator. However, it could be speculated that during the predation process alterations might cause a change in host specificity. A breach in host specificity could be undesirable, as it might allow the predators to attack communal non-pathogenic Gram-negative bacteria. In order to examine if a breach or alteration in predator host specificity could develop, predation resistant bacteria were used, and an attempt was made to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria. We have used both a Gram-negative and Gram-positive bacteria for this study. The data obtained suggests that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This was true for both *S. maltophilia* and *S. epidermidis* host cells. We also did not obtain any *M. aeruginosavorus* isolates that breached their host specificity and attacked previously resistant bacteria.

In an attempt to enrich for *B. bacteriovorus* hyper virulent isolates, we found that *B. bacteriovorus* 109J could become more virulent on particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria and not others. Additionally, continuous predation cycles did not seem to increase the ability *M. aeruginosavorus* to reduce a predation tolerant host. Finally, we were able to enrich for predators that were acclimated to attack at elevated temperatures.

Although the effect of predation on prokaryotic Gram-negative cells is documented, limited data is available regarding predation on eukaryotic cells. To determine if predatory bacteria have an adverse affect on eukaryotic cells, three human cell lines were exposed to high concentrations of predatory bacteria. Our data show that subjecting human epidermal keratinocytes, human skin fibroblasts and murine fibroblasts to the predators have caused no measurable toxic effect as compared to the control. Further more no cell detachment or morphological changes were seen in cells which were incubated with the predators. When measuring the cytokine levels in cells that were exposed to the predators we have seen that predatory bacteria did not provoke an elevated inflammation respond in human epidermal keratinocytes, however, it did provoke an inflammation respond in fibroblasts. This might be explained by the ability of some cells to better regulate their inflammation response in the presence of LPS. However at this point we can only conclude that the inflammation response is cell specific and while some cells will be stimulated by the predators other cell lines might be provoked.

Finally, when administered in vivo, predatory bacteria had showed no negative effect on the wellbeing of wounded mice. This was also correct when immunocompromised mice were used. The introduction of the predators to open wound had caused no significant difference with respect to weight loss, wound clinical score and wound time to close as compared to the non-inoculated control. This is the first time to our knowledge that predatory bacteria are administered to wounded and immunocompromised animals. At this point of the study we verified that predatory bacteria are safe when applied topically to open wounds. In the next part of the study we will determine if predatory bacteria are effective in reducing an active wound infection and supporting better wound healing.